REMARKS

Claims 1-32 are pending in the subject application. The Examiner stated that claims 8, 10, 12, 31 and 32 are withdrawn from consideration. Applicants have hereinabove cancelled claim 25 without prejudice or disclaimer to their right to pursue the subject matter of this claim in a later-filed application. In addition, applicants have hereinabove amended claims 1-3, 5-6, 13, 15-17, 19 21-24 and 26-30 and added new claims 33-43. Support for these amendments may be found inter alia in the specification as follows: claim 33: page 11, lines 6-23; claim 34: page 12, lines 6-16; claim 35: page 13, line 19-31; claim 36: page 13, lines 4-18; claim 37: page 11, line 24 - page 12, line 5; claim 38: page 12, lines 6-16; claim 39: page 13, lines 19-31; claim 40: page 12, lines 21-24; claim 41: page 8, line 16 - page 9, line 2; claim 42: page 11, lines 6-23; and claim 43: page 10, lines 16-24 and page 11, lines 6-23. The remaining changes to the claims merely introduce minor grammatical and format changes. In making these amendments, applicants neither concede the correctness of the Examiner's rejections in the March 18, 2004 Office Action, nor abandon their right to pursue in a continuing application embodiments of the instant invention no longer claimed in this application. These amendments do not involve any issue of new matter. Therefore, entry of this Amendment is respectfully requested such that claims 1-7, 9, 11, 13-24, 26-30 and 33-43 will be pending and under examination.

In view of the arguments and amendments set forth below, applicants maintain that the Examiner's objections and

rejections have been overcome and respectfully request that the Examiner reconsider and withdraw same.

Election/Restrictions

The Examiner acknowledged applicant's election with traverse of Group I, claims 1-7, 9, 11, 13, 15-19, and 21-30 in the paper filed December 22, 2003. The Examiner stated that the traversal is on the ground(s) that Groups I-VIII have overlapping claims, and that the differences in the groups appear to be a particular type of cell (response, page 6, 1st full paragraph). The Examiner stated that this is not found persuasive because the cell types of the different groups are special technical features that are not shared with each other. The Examiner stated that further, considerations, such as the operability of the silencing system within a cell type, are also different for the different cell types. The Examiner stated that applicants also argue that Groups IX-X are drawn to method of using the silencing system (response, paragraph bridging pages 6-7). The Examiner stated that during the course of examination, it was determined that it would not be an undue burden to further examine claim 20. The Examiner stated that Group IX was therefore rejoined with Group I. The Examiner stated that however, the method of Group X is distinct from the method of Groups I and IX. The Examiner stated that the method of Group X involves the use of random nucleic acid sequences, whereas the method of Group I uses nucleic acid sequences that has identity to a pre-determined target gene. The Examiner stated that applicants also argue that it would not be a serious burden to examine all groups together, and that a search of the prior art for Groups II-X would not be a serious burden once the prior art for Group I is

identified (response, page 7, 2nd full paragraph). The Examiner stated that however, as discussed above, the consideration of other issues, such as the operability of the system in the invention of Groups II-VIII and X, would be a serious burden.

The Examiner stated that Groups I and IX have been rejoined. The Examiner stated that it was also determined during the course of examination that it would not be an undue burden to further examine claim 14. The Examiner stated that claims 1-7, 9, 11, and 13-30 have been examined in this Office action. The Examiner stated that the restriction requirement of Groups II-VIII and X is still deemed proper and is therefore made final. The Examiner stated that applicants are reminded to remove non-elected subject matter from the elected claims.

In response, applicants respectfully traverse. Applicants acknowledge that the Examiner has rejoined the claims of Groups I and IX and examined claim 14. However, applicants maintain that claims 8, 10, 12, 31 and 32 are related and therefore not "independent". Therefore, it would not be a burden on the Examiner to examine claims 8, 10, 12, 31 and 32 in the subject application. Accordingly, applicants respectfully request that the Examiner reconsider and also rejoin claims 8, 10, 12, 31 and 32.

Information Disclosure Statement

The Examiner stated that the citation of the reference authored by Cox et al., Exhibit 12, was lined through in the IDS submitted March 12, 2002 because a date is not present (citing 37 C.F.R. §1.98 (b)(5)).

In response, applicants respectfully traverse. Nevertheless, without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application, applicants attach hereto as **Exhibit A** a PTO-1449 Form which sets forth the Cox et al. reference including its publication date. A copy of the Cox et al. reference including its publication information is attached thereto as **Exhibit 1**. Applicants respectfully request that the Examiner consider the attached reference and that this reference be printed on any patent which issues from the subject application.

Claim Objections

The Examiner objected to claim 2 because the term "said the" in line 6 should be deleted.

The Examiner objected to claim 5 because the term, "is" appears to be missing in line 2 after "sequence".

The Examiner objected to claim 21 because the article "a" appears to be missing in the first line before "plant".

The Examiner objected to claim 29 because the term "to" appears to be missing in line 2, after "corresponds".

The Examiner objected to claim 6 under 37 C.F.R. §1.75(c) for allegedly being of improper dependent form for failing to further limit the subject matter of a previous claim. The Examiner requires applicants to cancel the claim, or amend the claim) to place the claim in proper dependent form. The Examiner stated that the claim attempts to limit

the terminators of the silencing system of claim 1 or 2. The Examiner stated that however, claim 16 recites, "or any other suitable terminator capable of terminating the transcription...." The Examiner stated that this recitation does not limit the terminator of the parent claims.

Applicants note that the Examiner has referred to both claims 6 and 16 in the above objection. However, it appears that the objection should be directed to claim 16 and applicants treat this objection as such.

The Examiner objected to claim 7 under 37 C.F.R. §1.75(c) as allegedly being of improper dependent form for failing to further limit the subject matter of a previous claim. The Examiner required applicants to cancel the claim, or amend the claim to place the claim in proper dependent form, or rewrite the claim in independent form. The Examiner stated that the claim attempts to limit the T7 promoter of claim 1 or 2 by indicating that it corresponds to the promoter sequence of the bacteriophage T7 or functional analogues thereof. The Examiner stated that the specification defines pT7 as being from bacteriophage T7, and therefore this recitation does not limit claim 1 or 2. The Examiner stated that the parent claims 1 and 2 do not mention any functional analogues of pT7. The Examiner stated that if the silencing system of claim 17 does not comprise the pT7 promoter, then it does not comprise all of the limitations of the claims from which it depends.

Applicants note that the Examiner has referred to both claims 7 and 17 in the above objection. However, it appears that the objection should be directed to claim 17 and

applicants treat this objection as such.

In response, applicants respectfully traverse. Nevertheless, without conceding the correctness of the Examiner's objections but to expedite prosecution of the subject application, applicants have hereinabove amended claims 2, 5, 16, 17, 21 and 29 to address the claim language objected to by the Examiner.

The Examiner also objected to claim 26 under 37 C.F.R. §1.75(c) as being in improper form because a multiple dependent claim cannot depend from another multiple dependent claim. The Examiner stated that, accordingly, the claim has not been further treated on the merits.

In response, applicants respectfully traverse. Applicants note that claim 26 is dependent on claims 24 or 25, each of which is only dependent on claim 22. Therefore, applicants maintain that claim 26 is not dependent on a multiple dependent claim and satisfies the requirements of 37 C.F.R. §1.75(c).

In view of the above remarks, applicants respectfully request that the Examiner reconsider and withdraw the various grounds of objection.

Claim Rejections Under 35 USC §112, Second Paragraph

The Examiner rejected claims 1-7, 9, 11, and 13-30 under 35 U.S.C. §112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention.

The Examiner rejected claims 1-3, 5, 19-22, and 24-29 because the term "substantially" allegedly renders the claims indefinite. The Examiner stated that it is not clear what is meant by this recitation. The Examiner stated that the term is a relative term that has no definite meaning, and makes the metes and bounds of the claims unclear.

The Examiner stated that further in claim 1 the recitation, "a functional part thereof" in the last line renders the claim indefinite. The Examiner stated that the entire RNA or RNA transcript encodes the product of the target sequence. The Examiner stated that the specification, as discussed below, indicates that the silencing is occurring at the RNA level, that RNA transcripts of the target sequence cannot be detected. The Examiner stated that if the RNA transcript is cleaved, then the transcript will not produce its encoded product. The Examiner stated that it is then not clear what meant by the disappearance of "the functional part" of the RNA transcript, as opposed to the RNA transcript itself. The Examiner stated that it is suggested that the recitation be deleted from the claim, as it does not further define the invention and only adds confusion.

The Examiner stated that in claims 1, 2, 20, 21, 27, and 28 the recitation, "corresponding to the T7 RNA polymerase gene" renders the claims indefinite. The Examiner stated that it is not exactly clear what is meant by "corresponding." The Examiner suggested that the recitation, "a nucleotide sequence corresponding to" be deleted.

The Examiner stated that in claim 5 the recitation, "identical or homologous" renders the claim indefinite. The Examiner stated that it is not exactly clear what the difference between the two terms is, in the context of the targeting sequence and target sequence. The Examiner stated that the silencing system of the invention silences target sequences that share identical sequences with the targeting sequence. The Examiner stated that it is not clear if applicants intend "homologous" sequences to refer to differences between the target sequence and targeting sequence due to genetic code degeneracy. The Examiner stated that it is suggested that "or homologous" be removed from the claims.

The Examiner stated that in claim 6 the recitation "the silencing of which is desired" renders the claim indefinite. The Examiner stated that it is not clear how this recitation aids in defining the claimed invention. The Examiner stated that a gene that one desires to silence may not be desired by another. The Examiner suggested that the recitation be deleted.

The Examiner stated that in claims 6, 23, and 30 the recitation "target sequence corresponds to" renders the claims indefinite. The Examiner stated that it is not exactly clear what "corresponds" refers to. The Examiner stated that the metes and bounds of the claims are unclear. The Examiner suggested that "corresponds to" be replaced by "is".

The Examiner stated that further in claims 6, 23, and 30

the recitation "or to a fragment thereof, within the scope of degeneracy of the genetic code" renders the claim indefinite. The Examiner stated that it is not clear what the recitation is attempting to limit. The Examiner stated that it is not clear what genetic code degeneracy has to do with the fragment. The Examiner stated that it is also noted that, parts a) and b) encompass differences due to genetic code degeneracy, since these parts encompass all genes encoding all proteins, peptide products, and non-coding sequences.

The Examiner stated that further in claims 6, 23, and 30, part d) of the claims render them indefinite. The Examiner stated that part d) limits the target sequence to correspond to a nucleic acid sequence that hybridizes to any of the sequences of a)-c). The Examiner stated that however, parts a)-c) encompass any gene encoding any protein or peptide product, any non-coding sequence, and any fragment thereof within the scope of genetic code degeneracy. The Examiner stated that it is not clear how nucleic acid sequences of part d) are different from parts a)-c), or what nucleic acid sequences are encompassed by part d) that are not encompassed by parts a)-c). The Examiner stated that the metes and bounds of the claims are unclear.

The Examiner stated that in claim 13 the term, "optionally" renders the claim indefinite. The Examiner stated that it is not clear whether or not the additional regulatory elements are present in the claimed invention. The Examiner stated that if they are not, then the claim does not further limit claim 5. The Examiner suggested that the term

be deleted.

The Examiner stated that in claim 15 the recitation, "the plant promoter is p35S" renders the claim indefinite. The Examiner stated that the specification indicates that "p35S" is the CaMV 35S promoter (page 16,1st full paragraph). The Examiner stated that p35S is not a plant promoter. The Examiner suggested that the term, "plant" be deleted from the claim.

The Examiner stated that in claim 16 the claim introduces a narrowing limitation, "said terminators is the NOS terminator or a functional equivalent or fragment thereof, the B-1,3-gluconase terminator", but then recites a broader limitation "or any other suitable terminator...". The Examiner stated that a broad range or limitation together with a narrow range or limitation that falls within the broad range or limitation (in the same claim) is considered indefinite, since the resulting claim does not clearly set forth the metes and bounds of the patent protection desired. The Examiner noted the explanation given by the Board of Patent Appeals and Interferences in *Ex parte Wu,* 10 USPQ2d 2031, 2033 (Bd. Pat. App. & Inter. 1989), as to where broad language is followed by "such as" and then narrow language. The Board stated that this can render a claim indefinite by raising a question or doubt as to whether the feature introduced by such language is (a) merely exemplary of the remainder of the claim, and therefore not required, or (b) a required feature of the claims. Note also, for example, the decisions of *Ex parte Steigewald,* 131 USPQ 74 (Bd. App. 1961); *Ex parte Hall,* 83 USPQ 38 (Bd. App. 1948); and *Ex parte Hasehe,* 86 USPQ 481

(Bd. App. 1949).

The Examiner stated that in claim 17 the recitation, "corresponds" renders the claim indefinite. The Examiner stated that it is not exactly clear what the meaning of this recitation is. The Examiner suggested that the recitation, "corresponds to" be deleted.

The Examiner stated that in claims 22 and 29 the recitation, "substantially corresponds" renders the claim indefinite. The Examiner stated that it is not clear what targeting sequences are encompassed by this recitation. The Examiner stated that the metes and bounds of the claim are unclear.

The Examiner stated that in claim 25 the recitation, "carrying and expressing said silent target sequence" renders the claim indefinite. The Examiner stated that it is not clear what is meant by "carrying". The Examiner stated that the recitation also does not clearly indicate whether or not the target sequence is silent.

In response, applicants respectfully traverse the Examiner's above rejection. Nevertheless, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove cancelled claim 25 without prejudice or disclaimer to their right to pursue this claim in a later-filed application.

In addition, without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application, applicants have hereinabove amended

claims 1-7, 9, 11, 13-24 and 26-30 to address the claim language objected to by the Examiner.

In view of the above remarks, applicants contend that claims 1-7, 9, 11, 13-24 and 26-30 satisfy the requirements of 35 U.S.C. §112, second paragraph. Accordingly, applicants respectfully request that the Examiner reconsider and withdraw the various grounds of rejection.

Claim Rejections Under 35 U.S.C. §112, First Paragraph

Written Description

The Examiner rejected claims 1-7, 9, 11, 13-25, and 27-30 under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the written description requirement. The Examiner stated that the claims contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention.

The Examiner stated that the claims are broadly drawn towards an expression silencing system comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene or to a functional equivalent or fragment thereof which carry an NLS sequence, and a T7 promoter sequence or functional fragment thereof and at least one targeting sequence downstream of said pT7, which system is capable of rendering, upon introduction into a plant cell, the expression at the RNA level of a target sequence substantially silenced by causing the disappearance of the RNA or RNA transcript carrying said sequence or functional

part thereof; or wherein said target sequence corresponds to a) a gene encoding any protein or peptide product, b) a non-coding nucleic acid sequence, c) a nucleic acid which corresponds to a) or b) or a fragment thereof, within the scope of genetic code degeneracy, or d) a nucleic acid which hybridizes with the sequence according to a), b), or c) or a fragment thereof; or wherein said pT7 corresponds to the bacteriophage T7 promoter or functional analogues thereof; or wherein terminator sequences of said silencing system is the NOS terminator or a functional equivalent or fragment thereof; or a process for the transformation of a plant with said silencing system; or a method of silencing the expression of a target sequence within the genome of a plant, comprising said silencing system.

The Examiner stated that the specification indicates that the gene for the bacteriophage T7 RNA polymerase was fused to the translation enhancer element from Tobacco Mosaic Virus and a nuclear localization signal from SV40. The Examiner stated that this construct was operably linked to the CaMV 35S promoter and NOS terminator, and named "35S-T7-pol." The Examiner stated that two constructs comprising the GUS coding sequence were made, both comprising said translation enhancer element. The Examiner stated that in one construct, the GUS coding sequence was fused to the 35S promoter and the NOS terminator, and the other comprised the T7 promoter and both the NOS terminator and T7 terminator. The Examiner stated that Figures 1A and IB show diagrams of the constructs, and Figure 1C shows a diagram of a control construct comprising the GUS coding sequence fused to the 35S promoter and NOS terminator (page 16). The Examiner stated that the constructs were introduced into different

tobacco or tomato plants via Agrobacterium, and homozygous plants were selected at the R2 generation. The Examiner stated that transgenic plants comprising the T7 RNA polymerase construct, and which are capable of expressing GUS, were crossed with the transgenic plants comprising the T7-GUS constructs. The Examiner stated that the hybrid plants were self-pollinated and progeny selected for several generations. The Examiner stated that a 35S-T7-pol/pT7-GUS plant was pollinated with a plant expressing 35S-GUS. The Examiner stated that three of the eighteen progeny plants comprised all three constructs, none of which expressed GUS. The Examiner stated that GUS expression also was not detected in the 35S-T7-pol/pT7-GUS plants, except for in the pollen grain and callus of two plants. The Examiner stated that GUS was not expressed, however, in the leaves of those two plants. The Examiner stated that nuclear run-on assays and RNAse protections assays indicated that GUS was being transcribed, but GUS mRNA was not accumulating in the cytoplasm (pages 18-20). The Examiner stated that transgenic plants carrying the 35S-GUS construct were also crossed with the pT7-GUS plants, and the hybrid progeny selfed for several generations. The Examiner stated that a scion from a plant expressing the 35S-GUS construct was grafted onto the 35S-GUS/pT7-GUS silenced plants. The Examiner stated that shoots growing from 3 of 6 grafter scions silenced for GUS (pages 20-21).

The Examiner stated that the claimed expression silencing system comprises nucleotide sequences corresponding to the T7 RNA polymerase gene or to a functional equivalent or fragment thereof. The Examiner stated that a review of the full content of the specification indicates that nucleotide

sequences encoding the T7 RNA polymerase are essential to the operation of the invention.

The Examiner stated that the specification discloses only nucleotide sequences encoding the T7 RNA polymerase. The Examiner stated that nucleotide sequences encoding functional equivalents or fragments of T7 RNA polymerase are not described. Citing the *University of California v. Eli Lilly*, 119 F.3d 1559,43 USPQ 2d 1398 (Fed. Cir. 1997), the Examiner stated that the specification does not provide any evidence on the record of a relationship between the structure of T7 RNA polymerase-encoding nucleotide sequences and the structures of functional equivalents or fragments thereof. The Examiner stated that no information is provided indicating the portions of T7 RNA polymerase that are essential to its functional activity, or the changes the can be made to the sequence of T7 RNA polymerase that would leave its RNA polymerase activity intact.

The Examiner stated that the claimed expression silencing system also comprises nucleotide sequences a pT7 sequence or any functional fragment or functional equivalent or analogue thereof.

The Examiner stated that the specification, however, does not disclose any functional fragments or functional equivalents or analogues of pT7. The Examiner stated that the specification does not describe any relationship between the structure of pT7 and any fragments thereof that retain its promoter activity. The Examiner stated that no information is provided that indicates the sequences of pT7 that are essential to its activity, or how the sequences of

pT7 can be changed without affecting its activity.

The Examiner stated that claim 16 also recites that the silencing system comprises the NOS terminator or a functional equivalent or fragment thereof. The Examiner stated that the specification, however, does not describe any functional equivalents of the NOS terminator, or any fragments thereof that retains its activity. The Examiner stated that the specification is silent as to the sequences of the NOS terminator that are essential to its function, and does not describe sequences that can be changed without changing its activity. The Examiner stated that given the breadth of the claims and the description in the specification of only nucleotide sequences encoding T7 RNA polymerase pT7, and the NOS terminator, it is submitted that the specification fails to provide an adequate written description of the multitude of nucleotide sequences and transgenic plants encompassed by the claims.

In response, applicants respectfully traverse the Examiner's above rejection. Nevertheless, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove cancelled claim 25 without prejudice or disclaimer to their right to pursue this claim in a later-filed application.

In addition, without conceding the correctness of the Examiner's rejection, applicants have hereinabove amended claims 1, 2, 16, 20, 21, 27 and 28 so that they no longer recite "or to a functional equivalent or fragment thereof."

In view of the above remarks, applicants contend that claims

1-7, 9, 11, 13-24 and 27-30, as amended, satisfy the written description requirement of 35 U.S.C. §112, first paragraph. Accordingly, applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Enablement

The Examiner rejected claims 1-7, 9, 11, 13-25, and 27-30 under 35 U.S.C. §112, first paragraph, because the specification, while being enabling for an expression silencing system comprising a nucleotide sequence encoding the T7 RNA polymerase, and pT7, allegedly does not reasonably provide enablement for functional equivalents or fragments of nucleotide sequences encoding T7 RNA polymerase, pT7, and the NOS terminator; the expression silencing system wherein the target sequence is a non-coding sequence in the plant genome. The Examiner stated that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with these claims.

The Examiner stated that the claims are broadly drawn towards an expression silencing system comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene or to a functional equivalent or fragment thereof which carry an NLS sequence, and a T7 promoter sequence or functional fragment thereof and at least one targeting sequence downstream of said pT7, which system is capable of rendering, upon introduction into a plant cell, the expression at the RNA level of a target sequence substantially silenced by causing the disappearance of the RNA or RNA transcript carrying said sequence or functional

part thereof; or wherein said target sequence corresponds to a) a gene encoding any protein or peptide product, b) a non-coding nucleic acid sequence, c) a nucleic acid which corresponds to a) or b) or a fragment thereof, within the scope of genetic code degeneracy, or d) a nucleic acid which hybridizes with the sequence according to a), b), or c) or a fragment thereof; or wherein said pT7 corresponds to the bacteriophage T7 promoter or functional analogues thereof; or wherein terminator sequences of said silencing system is the NOS terminator or a functional equivalent or fragment thereof; or a process for the transformation of a plant with said silencing system; or a method of silencing the expression of a target sequence within the genome of a plant, comprising said silencing system.

The Examiner stated that the specification teaches the use of the T7 RNA polymerase/pT7 expression system in plants to silence a target sequence therein, as discussed above. The Examiner stated that however, the specification does not teach functional equivalents or fragments of nucleotide sequences encoding the T7 RNA polymerase, pT7, or NOS terminator. The Examiner stated that the specification makes no mention of any functional equivalents of any of these elements. The Examiner stated that the specification and prior art are silent as to the sequences of any of these elements that can be changed without affecting their respective functional activities. The Examiner stated that even minor changes in promoter sequences, for example, can have a drastic negative effect on its activity. The Examiner stated that Kim et al. (*Plant Mol. Biol.*, 1994, Vol. 24, pages 105-117), for example, teach that deletions of a few nucleotides have a negative effect on the functional

activity of the NOS promoter (pages 111-112). The Examiner stated that in the absence of further guidance, undue experimentation would be required by one skilled in the art to determine how the T7 RNA polymerase, pT7 promoter, and NOS terminator can be changed without affecting their functional activities.

In response, applicants respectfully traverse the Examiner's above rejection. Nevertheless, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove cancelled claim 25 without prejudice or disclaimer to their right to pursue this claim in a later-filed application.

In addition, without conceding the correctness of the Examiner's rejection, applicants have hereinabove amended claims 1, 2, 16, 20, 21, 27 and 28 so that they no longer recite "or to a functional equivalent or fragment thereof." Therefore, the Examiner's rejection based on this phrase is now moot.

In addition, the Examiner stated that the specification also does not enable the claimed invention when the target sequence is a non-coding sequence. The Examiner stated that the specification admits that the silencing induced by the claimed invention occurs at the RNA level, that the expression system allows transcription of the targeting sequence but that mRNA of the targeting sequence does not accumulate (page 9, 3rd full paragraph; pages 16-17). The Examiner stated that post-transcriptional gene silencing affects the expression of transgenes and endogenous genes with which they share a high degree of sequence identity.

The Examiner stated that as the silencing is due to homology shared between transcribed sequences, it is not clear, and not taught in the instant specification, how a non-coding sequence can be targeted with the claimed silencing system. The Examiner stated that in the absence of further guidance, undue experimentation would be required by one skilled in the art to determine how to use the claimed silencing system, which acts at the RNA level, to silence the expression of a target gene using non-coding nucleotide sequences. The Examiner cited *Genentech, Inc. v. Novo Nordisk, A/S*, 42 USPQ2d 1001, 1005 (Fed. Cir. 1997), which teaches that "the specification, not the knowledge of one skilled in the art" must supply the enabling aspects of the invention. The Examiner stated that given the breadth of the claims, unpredictability of the art, and lack of guidance of the specification as discussed above, undue experimentation would be required by one skilled in the art to make and use the claimed invention.

In response, applicants respectfully traverse. Applicants contend that shared homology can exist between a targeted non-coding sequence downstream the T7 promoter and, for instance, a translation regulatory element present at the 5'UTR and/or 3'UTR of RNA transcripts. Applicants direct the Examiner's attention to Example 5 in the subject specification, where the translational enhancer element Ω of TMV (as described by Gallie et al., *Nucleic Acids Res.* (1988), 16(17): 8675-8694), attached to the T7 promoter, induced resistance to the TMV virus in the transfected plants.

Example 5 of the subject specification describes how the use

of the TMV translation enhancer Ω element, a non-coding sequence, can be efficiently used as the targeting sequence in the silencing system described by the invention. Cloning of the 68 nucleotide TMV Ω element sequence downstream the T7 promoter and its transformation to a plant expressing the T7 RNA polymerase, conferred resistance to TMV infection. As shown in the Table 2 of the subject specification, the virus titer (as measured by ELISA) was significantly reduced in the plants double transformed with the silencing system.

In further support of their position, applicants submit a Declaration Under 37 C.F.R. §1.132 (**Exhibit B**) in which Dr. Ilan Sela declares that these results stem from the inability of the virus to replicate as shown in Figure 1 of Appendix A of her Declaration. Dr. Sela further declares:

> "6. These results stem from the inability of the virus to replicate as shown in Figure 1 of Appendix A (attached hereto as **Exhibit 2**). Dot blot analysis of TMV-infected protoplasts generated from non-transgenic plants and from silenced plants (using a TMV probe sequence) clearly shows increasing viral amounts in the non-transformed samples 48 hours post-infection. In the double transformed protoplasts, the signal remained at background level even after 72 hours post-infection, proving that the virus did not replicate and that the silencing effect at the cellular level is instantaneous.
>
> 7. Furthermore, TMV resistance induced by the

silencing system of the claimed invention prevails over time (11 weeks) as shown in Figure 2 of Appendix A (attached hereto as **Exhibit 2**).

8. At the plant level, following a brief stage of cellular silencing, the virus is able to overcome silencing by exerting its silencing suppressor capacity and replicate normally for a certain period (2-7 weeks). Later on, the plant's silencing mechanism overcomes the virus' effect and the viral infection declines. This plant phenomenon is acknowledged as "viral infection recovery."

9. Therefore, the data provided in the specification as filed and in Appendix A show that silencing can be induced by the system of the invention, using a non-coding nucleotide sequence as the targeting sequence.

10. The silencing induction with the subject system is very efficient at the cellular level and it persists over time.

11. Further experiments performed also show the claimed silencing system as versatile and very efficient in addition to being enabled. According to the data provided in Appendix B (attached hereto as **Exhibit 3**), T7-derived RNA-silencing of endogenous genes in plants can be achieved with 80-100% efficiency, when using even gene fragments (300-400 bp).

12. Silencing can be attained by transforming one group of plant cells with a first construct harboring the gene for T7-pol, transforming another group of plant cells with a second plasmid carrying even part of a target gene placed between pT7 and the T7 and *nos* terminators, crossing transformed plants from the first group with transformed plants from the second group and selecting for double transformed progeny (as demonstrated in the Tomato (*Lycopersicon esculentum*) example in Appendix B attached hereto as **Exhibit 3**).

13. Silencing can also be attained by transforming plant cells with the plasmid carrying the gene for T7 RNA polymerase (T7-pol) placed between the 35S constitutive promoter and the *nos* terminator. Plants expressing the *T7-pol* are re-transformed with another plasmid carrying a nucleotide targeting sequence placed between the T7 promoter (pT7) and the T7 and the *nos* terminators, and double-transformed plants are selected (as demonstrated in the Tobacco *Nicotiana N* gene silencing example in Appendix B attached hereto as **Exhibit 3**).

14. Using both approaches, gene silencing was efficiently accomplished.

15. When *V. dahliae*-resistant, non-transformed VF-36

plants and doubly-transformed VF-36 tomato plants were challenge-inoculated with a virulent isolate of *V. dahliae* race 1, the doubly transformed VF-36 plants lost their resistance. The transformed plants became *Verticillium*-susceptible and remained stunted, most of them eventually dying. Tomato plants, silenced for *Ve*1, lost their resistance to *Verticillium*. These phenotypic changes were noted in 100% of the *Ve*-silenced tomato plants.

16. Tobacco plants carrying the *N* gene (tobacco^{NN}) react to TMV infection by producing local necrotic lesions restricted to sites around the point of virus entry. When leaves of the doubly transformed plants, silenced for the N gene were inoculated with TMV, necrosis spread beyond the local lesions in about 80% of the transformed plants. TMV did not spread and remained confined to the site of infection. The T7-derived silencing system lead to a phenotypic change with respect to the spread of necrosis.

17. T7-derived RNA-silencing of endogenous *Ve*1 and *N* genes was not only detected at the molecular level, but was also manifested by phenotypic changes.

18. The T7-derived silencing system is not limited to transgenes such as *GUS* or exogenous pathogens such as TMV. The T7-derived silencing system also successfully silenced endogenous genes like the

tobacco *N* gene and the tomato *Ve*1 gene."

Therefore, applicants maintain that the data provided in the subject specification and in Dr. Sela's Declaration demonstrate that silencing can be induced by the system of the invention by using a non-coding nucleotide sequence as the targeting sequence. Thus, applicants contend that the application as filed does provide the teachings which would enable one of ordinary skill in the art, using routine experimentation, to use a non-coding sequence as the targeting sequence in the present invention.

In view of the above remarks, applicants contend that claims 1-7, 9, 11, 13-24 and 27-30, as amended, satisfy the enablement requirement of 35 U.S.C. §112, first paragraph. Accordingly, applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Rejections Under 35 U.S.C. §102(b)

The Examiner rejected claims 1-7, 13-15, 17, and 19 under 35 U.S.C. §102(b) as allegedly being anticipated by Lassner et al.

The Examiner stated that the claims are broadly drawn towards an expression silencing system comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene or to a functional equivalent or fragment thereof which carry an NLS sequence, and a T7 promoter sequence or functional fragment thereof and at least one targeting sequence downstream of said pT7.

The Examiner stated that Lassner et al. teach a DNA

construct comprising the nucleotide sequence encoding the T7 RNA polymerase operably linked to the double CaMV 35S promoter, a nucleotide sequence encoding the SV40 nuclear locator signal, and a transcription terminator sequence, and a DNA construct comprising the T7 promoter operably linked to nucleotide sequences of interest and a terminator. The Examiner stated that the constructs taught by the reference comprise the limitations of the elements of the DNA construct products encompassed by the claims. The Examiner stated that the property of silencing a target sequence, when present in a cell, which shares sequence identity with the nucleotide sequence operably linked to the T7 promoter is inherent to the T7 RNA polymerase/T7 promoter system taught by the reference. The Examiner stated that claim 19 is included in this rejection, as it recites that the DNA constructs are "substantially" as shown in instant Figures 1A and IB. Given the indefiniteness of the recitation, "substantially" (see the rejection above), the DNA constructs of the reference are substantially the same as those shown in the instant figures.

In response, applicants respectfully traverse and maintain that Lassner et al. fail to teach each and every element of the rejected claims.

Lassner et al. teach the possibility of combining the use of the prokaryote T7 RNA polymerase gene together with plants normally functional regulatory elements for successful gene expression: "This system demonstrates the feasibility of T7 RNA polymerase-based approaches for the high-level expression of introduced genes in plant cells" (see abstract, page 229, last sentence).

Lassner et al. state in the introduction (page 239, line 12): "We have introduced T7 RNA polymerase genes into tobacco protoplasts for two purposes: (1) to study nuclear transport proteins in plant cells and (2) to develop a T7 RNA polymerase-mediated expression system for plants". Their objective was thus to achieve a functional T7 RNA polymerase driven expression system in plants.

Lassner et al. describe the use of a single construct capable of expressing an active prokaryote T7 RNA polymerase in plants. They demonstrated the T7 RNA polymerase activity by *in vitro* experiments: "T7 RNA polymerase activity was detected in extracts of protoplasts electroporated with both genes" (see abstract, page 229, line 4). From their experiments, it is not clear that successful transcription and proper translation may actually occur *in vivo* in plant cells.

For a person of skill in the art, the conclusion that can be reached from the Lassner et al. article is that a nucleotide sequence regulated by the T7 promoter should be over-expressed when transfected into plant cells already expressing the T7 RNA polymerase.

Although over-expression of a nucleotide sequence placed downstream of the potent and specific T7 promoter may be achieved. On the other hand, the T7 expression system should not necessarily interfere with the expression of endogenous promoters and genes. This possibility was never mentioned or implied by the Lassner et al. paper.

Lassner et al. conclude by saying: "These experiments are a step in the development of a T7 RNA polymerase-mediated gene expression system for plants" (see discussion, page 233, first line of last paragraph). Based on this sentence, the authors did not see any "inherent" potential of using this system for silencing gene expression. Quite the contrary.

Applicant maintains that the invention is novel over Lassner et al., by defining a new system comprised of a **double construct** (not a single construct as used by Lassner et al.). The introduction of the combined construct system into plant cells, results in the *in vivo* expression of the T7 RNA polymerase from the first construct and an active transcription, driven by the T7 promoter, of a nucleotide sequence present in the second construct.

Surprisingly, the **combined activity** of these constructs introduced into plant cells induces **gene silencing** of exogenous and endogenous genes and not over-expression as taught by Lassner et al.

In view of the above remarks, applicants maintain that claims 1-7, 13-15, 17, and 19 are not anticipated by Lassner et al. and satisfy the requirements of 35 U.S.C. §102. Therefore, applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Rejections Under 35 U.S.C. §103

The Examiner rejected claims 1-7, 9, 13-17, 19-25, and 27-30 under 35 U.S.C. §103(a) as allegedly being unpatentable over Lassner et al. in combination with Blokland et al. (Plant J., 1994, Vol. 6, pages 861-877), and Palauqui et

al.(EMBOJ., 1997, vol. 16, pages 4738-4745).

The Examiner stated that the claims are broadly drawn towards an expression silencing system comprising a nucleotide sequence corresponding to the T7 RNA polymerase gene or to a functional equivalent or fragment thereof which carry an NLS sequence, and a T7 promoter sequence or functional fragment thereof and at least one targeting sequence downstream of said pT7, which system is capable of rendering, upon introduction into a plant cell, the expression at the RNA level of a target sequence substantially silenced by causing the disappearance of the RNA or RNA transcript carrying said sequence or functional part thereof; or wherein said target sequence corresponds to a) a gene encoding any protein or peptide product, b) a non-coding nucleic acid sequence, c) a nucleic acid which corresponds to a) or b) or a fragment thereof, within the scope of genetic code degeneracy, or d) a nucleic acid which hybridizes with the sequence according to a), b), or c) or a fragment thereof; or wherein said pT7 corresponds to the bacteriophage T7 promoter or functional analogues thereof; or wherein terminator sequences of said silencing system is the NOS terminator or a functional equivalent or fragment thereof; or a process for the transformation of a plant with said silencing system; or a method of silencing the expression of a target sequence within the genome of a plant, comprising said silencing system. Lassner et al. is discussed above.

The Examiner stated that Lassner et al. do not teach crossing a first plant comprising a DNA construct comprising nucleotide sequences encoding the T7 RNA polymerase operably

linked to a NLS, with a second plant comprising a DNA construct comprising the T7 promoter operably linked to a targeting sequence, or the progeny of the cross comprising both constructs and in which a target sequence is silenced; or grafting.

The Examiner stated that Blokland et al. teach co-suppression of pigmentation of chalcone synthase (chs) in Petunia plants comprising a construct comprising the CaMV 35S promoter operably linked to a nucleotide sequence encoding a fusion of the uidA (GUS) gene and the chs A cDNA. The Examiner stated that steady-state chs mRNA levels, and pigmentation, were reduced in the transgenic plants, including in flowers. The Examiner stated that GUS expression was also silenced in plant parts that were also silenced for chs. The Examiner stated that the constructs used also comprised nos terminator operably linked to the uidA/chs coding sequence (pages 862-866).

The Examiner stated that Palauqui et al. teach that silencing is transmitted with 100% efficiency from silenced rootstock, derived from transgenic plants that were co-suppressed for nitrate reductase and the uidA transgene, to grafted scions that were from transgenic plants that nitrate reductase and uidA (pages 4739-4742).

In response to the Examiner's rejection, applicants respectfully traverse, and maintain that the Examiner has failed to establish a prima facie case of obviousness against the rejected claims.

To establish a prima facie case of obviousness, the Examiner

must demonstrate three things with respect to each claim. First, the cited references, when combined, teach or suggest each element of the claim. Second, one of ordinary skill would have been motivated to combine the teachings of the cited references at the time of the invention. And third, there would have been a reasonable expectation that the claimed invention would succeed.

Applicants contend that the references cited against the rejected claims fail to support a prima facie case of obviousness.

To support a case of prima facie obviousness, Lassner et al., Blokland et al., and Palauqui et al., when combined, would have to teach or suggest all elements of the rejected claims. Moreover, there would have to have been a motive to combine them, and a reasonable expectation of the invention's success at the time of the invention. This they fail to do.

In support of their position, applicants submit a Declaration Under 37 C.F.R. §1.132 in which Dr. Ilan Sela declares:

> "20. The claimed T7-derived silencing system holds features different from other silencing systems described in the cited literature, as detailed in Appendix C (attached hereto as **Exhibit 4**).
>
> 21. T7-derived silencing is a type of RNA silencing. However, several parameters associated with the silencing pathway in plants were not observed. No

siRNA could be detected in total RNA extracts of silenced plants. The silencing signal was not transduced across grafts. The viral silencing suppressor HC-pro could not overcome the T7-derived silencing effect. Yet, the silenced genes were methylated at their coding regions, pertinent siRNAs were detected only in nuclear extracts and dicer activity was enhanced in silenced plants. The T7-derived silencing system activity seems to be confined to the nucleus.

22. I understand that the Examiner asserts that Blokland et al. teach co-suppression of pigmentation of chalcone synthase (chs) in Petunia plants comprising a construct comprising the CaMV 35S promoter operably linked to a nucleotide sequence encoding a fusion of the uidA (GUS) gene and the chsA cDNA. Steady state chs mRNA levels, and pigmentation, were reduced in the transgenic plants, including in flowers. GUS expression was also silenced in plant parts that were also silenced for chs. The construct used also comprised nos terminator operably linked to the uidA/chs coding sequence (pages 862-866).

23. Certain aspects of the silencing process described by Blockland et al. are distinct from the process of the claimed invention. One of the most important is the difference in the efficiency of the system. While Blockland et al. show about 8% efficiency (1 out of 12-15), the T7-driven silencing system is 80-100% efficient (see Appendix B attached hereto

as **Exhibit 3**).

24. Blockland et al. demonstrate that silencing of the *chs* genes in petunia is not always associated with highly transcribed *chs* transgenes. This suggests that the absolute level of normal mRNA is not important. As shown in Appendix B (attached hereto as **Exhibit 3**), TMV infection considerably stimulates *N* expression in tobacco^{NN} and the degree of silencing was proportional to the level of the expressed *N mRNA*. Leaves with the highest levels of *N mRNA* exhibited the strongest silencing. The degree of RNA silencing is dependent of the level of the mRNA.

25. Blockland et al. state that the "suppressed *chs* genes and transgenes are transcribed seemingly unaltered; indicating that silencing does not result from a type of DNA modification that prevents transcription initiation." They also were not able to detect differences between the methylation status of suppressed and non-suppressed genes. As described in Appendix C (attached hereto as **Exhibit 4**), a difference in the *Hpa*II cleavage pattern was observed between silenced and non-silenced plants, indicating that a CCGG sequence at the NBS domain of *N* was methylated. Methylation density analysis and cytosine methylation test (using the bisulfite method) *in GUS*-silenced plants show a dense DNA methylation at the *GUS* coding region. In the silenced plants, cytosine

methylation was not restricted to GC or GNC sites.

26. Another unique characteristic of the T7-driven silencing system is the capacity of using only a short homology fragment (around 70 nucleotides) as the targeting sequence in order to successfully induce and preserve gene expression silencing as shown in the TMV translation enhancer Ω element experiments in Appendix A (see **Exhibit 2** attached hereto).

27. The Examiner states: "Palauqui et al. teach that silencing is transmitted with 100% efficiency from silenced rootstock, derived from transgenic plants that were co-suppressed for nitrate reductase and the uidA transgene, to grafted scions that were from transgenic plants that nitrate reductase and uidA (pages 4739-4742)."

28. In plants, usually long-distance silencing signals engender silencing in non-silenced scions upon grafting on silenced rootstocks as described in Palauqui et al.

29. In the T7-derived silencing system a lack of long-distance signaling was observed (see Appendix C, Table 1, attached hereto as **Exhibit 4**). Doubly-transformed plants, silenced for *GUS*, were grafted with scions harboring and expressing 35S-*GUS* without silencing *GUS* expression in the scions. Reciprocally, GUS expression also occurred when the

35S-*GUS* plants served as rootstocks on which the *GUS*-silenced doubly transformed plants were grafted. To rule out the possibility that lack of signaling between rootstock and scion was due to inept grafting technique, TMV-infected scions were grafted on healthy plants, and demonstrated the virus' spread across the graft. Also demonstrated was that in the case of intron-spliced hairpin silencing of *GFP*, a long-distance signal is transduced across the graft, engendering silencing in GFP-expressing scions (data not shown).

30. In the T7-driven silencing system, silencing is restricted to the cells carrying the constructs. There is no transmission of the signal to other tissues as seen in *C. elegance* or in plants, as described by Palauqui et al.

31. Finally, the Examiner asserts that "it would have been obvious and within the scope of one ordinary skill in the art at the time the invention was made to use the RNA polymerase/pT7 system of Lassner et al. to silence gene of interest in plant cells, for example the chs gene of Blockland et al. It was obvious that the constructs comprising the T7 RNA polymerase coding sequence, and pT7-target sequence could have been introduced into the same plant cell, and a transgenic plant regenerated therefrom; or into separate plant cell, wherein two transgenic plants would have been regenerated therefrom, and subsequently crossing the two plants to bring the two constructs into the same plant. Whether both

DNA constructs were introduced into the same plant initially, or into different ones which were subsequently crossed, amounts to an optimization of process parameters. It further would have been obvious that crossing the plant containing both DNA constructs to a non-transgenic plant that expresses the target sequence, would result in progeny plants that comprise both DNA constructs, and that the target sequence would have become silenced. It further would have been obvious to graft a plant, in which the target sequence, for example chs, expressed its product, onto a rootstock from the transgenic plant comprising the two DNA constructs and wherein the targeting sequence, for example, was from the chs coding sequence. It would have been obvious, given the teaching of Palauqui et al., that the chs coding sequence would have become silenced in the grafted scion. One would have been motivated to use the T7 RNA polymerase/pT7 system to express a target in sequence to silence a target sequence, given the teaching of Lassner et al. that this system successfully allows the transcription of nucleotide sequences operably linked to pT7 when in plant cells."

32. The T7-driven silencing system of the application comprises two constructs: T7 RNA polymerase and T7 promoter-target sequence. These two constructs acting together engender silencing of transgenes, endogenous and exogenous pathogenic genes. The silencing induction is very efficient (≅100%) and a small targeting sequence (as small as 70bp) is

> sufficient to induce and preserve gene expression silencing. This system's silencing process is a post-transcriptional event but also involves DNA methylation. Its activity is constrained to the transformed cells and it cannot be transmitted from stocks to grafted scions."

Given this information, it is clear that the T7-driven silencing system is not based on Lassner et al. in combination with Blokland et al. and Palauqui et al., but a new system mediated by a different mechanism.

Accordingly, the Examiner has failed to establish the prima facie obviousness of claims 1-16, 19-25, and 28-30 over these references.

In view of the above remarks, applicants maintain that claims 1-7, 9, 13-17, 19-24, and 27-30 satisfy the requirements of 35 U.S.C. §103(a). Accordingly, applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Summary

For the reasons set forth hereinabove, applicants respectfully request that the Examiner reconsider and withdraw the various grounds of objection and rejection and earnestly solicit allowance of the now pending claims, i.e. claims 1-7, 9, 11, 13-24, 27-30 and 33-43, and rejoinder of claims 8, 10, 12, 31 and 32.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants'

undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee, other than the $647.00 sum, which includes the $475.00 fee for a three-month extension of time and the $172.00 fee for additional claims, is deemed necessary in connection with the filing of this Amendment. However, if any additional fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,

John P. White
Registration No. 28,678
Attorney for Applicants
Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036
Tel. No. (212) 278-0400

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

9/17/04

John P. White Date
Reg. No. 28,678

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Examiner Initial		Document Number	Date	Name	Class	Subclass	Filing Date if Appropriate

FOREIGN PATENT DOCUMENTS

		Document Number	Date	Country	Class	Subclass	Translation	
							Yes	No

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

		Cox, K.H. et al. (1988) "Analysis of Plant Gene Expression" in Plant Molecular Biology, A Practical Approach (Shaw, C.H. ed., IRL Press), pages 1-35 **(Exhibit 1)**.

EXAMINER	DATE CONSIDERED

*EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP 609: Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Applicants: Ilan Sela et al.
Serial No.: 09/889,821
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Exhibit A

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Plant molecular biology

a practical approach

Edited by
C H Shaw
Department of Biological Sciences, University of Durham, Science Laboratories, South Road, Durham DH1 3LE, UK

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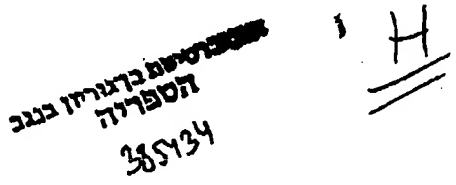

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Preface

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Most importantly, I hope that t
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others will gain from reading it.

Contents

CHAPTER 1

Analysis of plant gene expression

KATHLEEN H.COX and ROBERT B.GOLDBERG

1. INTRODUCTION

Our knowledge of the cellular processes that control plant gene expression has increased enormously during the last 5 years. This has been due in part to innovative technological advances in molecular biology and the application of these techniques to the analysis of plant gene structure and expression. In this chapter, we describe several qualitative and quantitative gene expression assays that are being used currently in our laboratory. We have concentrated on two types of techniques: those that are specific for plants, or have been optimized for plant systems; and those that are more recent and are not generally available. We have avoided discussion of many techniques pioneered in animal systems that are directly applicable to plant research such as the construction of cDNA libraries (1–3), gel electrophoresis techniques (4) and S1 and primer extension assays (5,6).

This chapter describes procedures for the following techniques.

(i) RNA isolation. In this section, we have provided procedures for isolating whole cell, nuclear, polysomal and poly(A^+) RNA.

(ii) Nuclei isolation and run-off transcription. This section describes how to isolate nuclei from a variety of organs. In addition, it provides procedures to label the pre-initiated nuclear RNA to measure the relative transcription rates of individual genes.

(iii) Synthesis of single-stranded RNA probes and use in RNA blot analysis. This section describes how to synthesize RNA probes from any of the commercially available bacteriophage promoter–RNA polymerase systems. These probes can be used for *in situ* hybridization, RNA titration analysis and RNase mapping. This section also contains detailed protocols for their use in blot analysis.

(iv) RNA titration analysis. RNA titration is the method of choice for determining the number of copies of a particular RNA sequence. This section provides detailed procedures for this technique, as well as the equations required to analyse the data.

(v) *In situ* hybridization. This section provides procedures for every aspect of *in situ* hybridization from tissue fixation through data analysis. Steps in the technique that are particularly sensitive to the organ system or tissue source are pointed out to aid in optimizing this procedure for other plant systems.

2. RNA ISOLATION

2.1 **Introduction**

A successful RNA purification technique yields undegraded and biologically active RNA that is uncontaminated with other macromolecules. Isolating intact plant RNA has been complicated by high levels of endogenous RNases in plant cells. RNA degradation during isolation has been inhibited by the use of a variety of RNase inhibitors including chelators such as EDTA and EGTA (7), detergents such as sodium dodecyl sulphate (8) and strong denaturants such as guanidinium isothiocyanate (9).

The choice of an extraction procedure will depend primarily on the fraction of RNA that is desired. This section provides procedures for isolating whole cell, nuclear, polysomal and poly(A^+) RNA.

Table 1. Total RNA extraction—guanidinium isothiocyanate.

Stock solutions

Solutions are filtered through 0.45 μm Millipore filters.

1. Guanidinium isothiocyanate extraction buffer
   4 M Guanidinium isothiocyanate
   0.5% Sodium lauroylsarcosinate
   50 mM Tris
   10 mM Na_2EDTA
   5 mM Sodium citrate
   0.1 M β-mercaptoethanol
   Adjust the pH to 7.0, sterile filter and store up to a month at room temperature.
2. 1 M Acetic acid
   Make this solution up in DEPC-treated (see Section 2.2.2) water.
3. Tris/Sarc
   10 mM Tris
   1% Sodium lauroylsarcosinate
   Adjust the pH to 8.0, treat with DEPC (see Section 2.2.2), filter and autoclave.
4. Tris/Sarc/CsCl
   10 mM Tris
   1% Sodium lauroylsarcosinate
   5.7 M CsCl
   Adjust the pH to 8.0.

Procedure

1. Grind the tissue to a fine powder in liquid nitrogen in a Waring blender[a].
2. Transfer the powder and liquid nitrogen to an omni-mixer cup and allow the liquid nitrogen to evaporate[b].
3. Add a 5× excess (v/w) of guanidinium isothiocyanate extraction buffer (0°C) to the powder (50 ml buffer/10 g tissue).
4. Homogenize in an omni-mixer on ice at top speed for 60 sec.
5. Spin the homogenate at 10 000 r.p.m. for 10 min using the SS34 Sorvall rotor at 4°C. This will pellet particulate material.
6. Measure the supernatant. Add 0.025 vols of 1 M acetic acid to lower the pH from 7 to 5. Add 0.75 vols of ethanol. Precipitate overnight at −20°C.
7. Spin the ethanol precipitate at 10 000 r.p.m. for 20 min in the SS34 Sorvall rotor at 4°C.
8. Pour off the supernatant and drain the tube. Wash the pellet with 70% ethanol and dry.

9. Resuspend the pellet in 8 ml of Tris/Sarc. Incubate on ice for 1 h. If there are problems resuspending the pellet, incubate it briefly at 68°C[c].
10. Pellet the insoluble material by spinning at 8000 r.p.m. for 20 min in the SS34 rotor at 4°C.
11. Measure the supernatant and bring it back up to 8 ml by adding Tris/Sarc.
12. Add 8 g of CsCl.
13. Add 1.25 ml of Tris/Sarc/5.7 M CsCl to three 5 ml ultracentrifuge tubes.
14. Overlayer the RNA/CsCl solution. Bring the solution to within ⅛ in of the top of the tube by adding Tris/Sarc.
15. Spin at 35 000 r.p.m. for 20 h in the AH650 Sorvall rotor at 20°C. This spin will pellet the RNA.
16. Remove the CsCl down to the cushion. Carefully and thoroughly wash the sides of the tube with DEPC-treated water[d]. Remove the CsCl cushion.
17. Wash the pellet with 70% ethanol and dry.
18. Resuspend the pellets in DEPC-treated water on ice for 1 h. Heat briefly to 68°C if necessary.
19. Spin out the insoluble material with a brief spin in a microfuge at 4°C.
20. Add ⅒ vol. of 3 M sodium acetate and 2 vols of ethanol.
21. Store at −20°C.

[a]Steps 1–4 should be done in a 4°C coldroom.
[b]When the liquid nitrogen is completely evaporated, the tissue will be lighter in colour and will not clump. It is important that the liquid nitrogen evaporate so that ice crystals do not form in the buffer. However, it is even more important that the tissue does not thaw.
[c]It is important at this step that the pellet be totally resuspended or the RNA yield will be low. Tissues that contain a large amount of starch or polysaccharides may result in large pellets which are difficult to resuspend.
[d]This step must be done carefully to prevent contamination of the RNA pellet by the supernatant.

2.2 General guidelines for RNA isolation

2.2.1 *Glassware*

All glassware and plastics should be dipped in water containing 0.02% diethylpyrocarbonate (DEPC). All items that can withstand the heat should be baked overnight at a temperature greater than 200°C or autoclaved. The rest should be dried overnight at room temperature.

2.2.2 *Solutions*

Most of the solutions (see individual notes) should be treated with 0.05% DEPC. DEPC is added to the solution, it is shaken vigorously and autoclaved for 30 min to break down the DEPC. The hot solution should be swirled after autoclaving to remove residual CO_2, a DEPC breakdown product.

2.2.3 *Plant harvesting*

If insects or eggs are present, the plant material should be rinsed with water before harvesting. It is then frozen in liquid nitrogen as soon as it is harvested and should not be allowed to thaw at any subsequent point. Plant material can be stored at −80°C for years without RNA degradation.

2.2.4 *Technique*

Gloves should be worn at all times. All solutions, rotors and glassware should be pre-cooled to 4°C before use. Stock solutions should be stored at −20°C between uses.

2.3 Whole cell RNA isolation

This total cellular RNA isolation procedure relies on a high concentration of the chaotropic agent, guanidinium isothiocyanate, in conjunction with the reductant β-mercaptoethanol. Guanidinium isothiocyanate rapidly denatures proteins resulting in dissociation of ribonucleoprotein complexes and inactivation of enzymes including RNases. The procedure shown in *Table 1* is a modification of the procedure of Chirgwin *et al.* (9) that we have used to isolate total RNA from tobacco roots and stems. These RNAs appear to be undegraded as analysed by denaturing gels, and they have been used for RNA blot analysis. We have not determined if this procedure results in RNA of sufficient purity to be used as a template for enzymatic reactions.

The cells are lysed in guanidinium isothiocyanate. After lysis there are two different procedures that can be used to isolate the RNA. One involves a series of precipitations in guanidinium hydrochloride (9) and the other, which is provided in our protocol, uses centrifugation of the RNA through a CsCl cushion to isolate the RNA from the rest of the cellular macromolecules (9).

The advantage in using this technique is that it is fast and results in undegraded RNA. The disadvantage of this technique is that we have observed that, depending on the source of the plant material, the pellet formed after the initial ethanol precipitation can be very hard to resuspend. When this occurs, the RNA yield is low.

2.4 Nuclear RNA isolation

Nuclear RNA can be isolated by the same procedure as outlined in Section 3.4. This technique has been used in our laboratory to isolate RNA for *in vitro* transcription and solution hybridization analysis. We have not used it for blot analysis and do not know if these RNAs are full length.

2.5 Polysomal RNA isolation

In order to analyse a mRNA population that is translated, as opposed to total cytoplasmic RNA, it is necessary to isolate polysomes and then release the mRNA from those structures. We have found that a modification of the Jackson and Larkins (7) procedure shown below results in high yields of undegraded polysomal mRNA. Stock solutions are given in *Table 2*.

This technique utilizes high pH, detergents, EGTA and β-mercaptoethanol to inhibit RNases during the lysis steps. The mRNA is separated from the cytoplasmic extract containing endogenous RNases by pelleting the RNA through a sucrose cushion. The pellet contains the polysomes along with some chromatin (a DNase step can be included in the protocol if the RNA must be

free of DNA) and membrane bound polysaccharides, while RNases remain in the supernatant. For this reason it is critical that steps in which the supernatant is removed (steps xi and xii) be done carefully so the pellet is not contaminated.

The first centrifugation spin (step vi) removes the nuclei from the cellular homogenate. Some of the nuclei lyse before this step, contaminating the subsequent polysomal RNA with nuclear RNA. If the polysomal RNA must be free of nuclear RNA, the EDTA-release procedure described in *Table 3* can be used (10,11). The rationale behind this procedure is that the mRNAs associated with polysomes will be released by the EDTA and be found in a fraction which sediments at less than 80S, while contaminating nuclear RNAs will be unaffected and sediment at greater than 100S. The level of contaminating nuclear RNA is low without the EDTA-release procedure. Therefore for general purposes, we do not include these steps.

The polysome profile (step xvi) can be used to assess the quality of the isolated RNA. Profiles vary slightly from organ to organ. However, on average, polysome preparations from organs other than leaf peak at 5-mers to 6-mers. Distinct peaks can be seen up to 10-mers. Leaf profiles peak at 7-mers to 8-mers with distinct peaks to 12-mers.

This technique has been used with minor modifications to isolate mRNA from various plants and organs. We have modified the extraction buffer slightly for use with different plants (see *Table 2*). mRNA isolated by this procedure has been used in a variety of techniques including blot analysis, S1 analysis, cDNA synthesis and R-loop analysis.

For the polysomal mRNA extraction carry out steps i–v and vii in the coldroom (all solutions are given in *Table 2*).

(i) Grind the tissue in liquid nitrogen in a Waring blender for 2 min or until the tissue becomes a uniform fine powder (see *Table 5* for tissue:buffer ratios).

(ii) Allow the liquid nitrogen to completely evaporate without thawing the tissue. Residual liquid nitrogen will cause formation of ice crystals and foaming during the homogenization step.

(iii) Add the powder to 200 ml of extraction buffer (0°C) in the omni-mixer cup.

(iv) Immerse the omni-mixer cup in ice and homogenize the tissue for 1.5 min at high speed.

(v) If the homogenate contains a large amount of debris, filter it through Nitex cloth (12 in × 12 in, 44 micron, monofilament, nylon mesh, Small Parts Inc., Miami, FL). Nitex should be DEPC-treated (Section 2.2.2) and autoclaved before use.

(vi) Spin the homogenate at 10 000 r.p.m. at 4°C in a Sorvall SS34 rotor for 20 min. This step removes debris, unlysed cells and organelles.

(vii) Carefully decant the supernatant into a glass beaker with a stir bar on ice. Add 20% detergent solution to a final concentration of 1%. Stir at moderate speed for 30 min. This step solubilizes membrane-bound polysomes.

Table 2. Stock solutions for polysomal RNA isolation.

Solutions are filtered through 0.45 μm Millipore filters and stored at −20°C.

1. 2× Salts for extraction buffer (see Section 2.5)

Tobacco extractions	Soybean extractions
0.4 M Tris	0.4 M Tris
0.2 M KCl	0.8 M KCl
0.05 M EGTA	0.05 M EGTA
0.07 M $MgCl_2 \cdot 6H_2O$	0.07 M $MgCl_2 \cdot 6H_2O$

   Adjust the pH to 9.0 with KOH, filter, treat with DEPC (see Section 2.2.2) and autoclave.
2. 10× Salts for sucrose cushion and resuspension buffer[a]
   0.4 M Tris
   2.0 M KCl
   0.05 M EGTA
   0.3 M $MgCl_2 \cdot 6H_2O$
   Adjust the pH to 9.0 with KOH, filter, treat with DEPC (see Section 2.2.2) and autoclave.
3. 10× Salts for analytical polysome gradients[a]
   0.4 M Tris
   0.2 M KCl
   0.1 M $MgCl_2 \cdot 6H_2O$
   Adjust the pH to 8.5 with HCl, filter, treat with DEPC (see Section 2.2.2) and autoclave.
4. 2 M Sucrose–DEPC
   Treat with DEPC (see Section 2.2.2) and autoclave.
5. 2 M Sucrose[b]
   Autoclave.
6. Detergents[c]
   20% Brij 35 (Sigma)
   20% Tween 40 (Sigma)
   20% Nonidet P-40 (BRL)
   Autoclave.
7. 2× Salts for proteinase K digestion (2× TES)
   20 mM Tris
   0.2 mM EDTA
   2% Sodium lauroylsarcosinate
   Adjust the pH to 7.6 with HCl, filter, treat with DEPC (see Section 2.2.2) and autoclave.
8. 3 M Sodium acetate
   Adjust the pH to 6.0 with glacial acetic acid, filter, treat with DEPC (see Section 2.2.2) and autoclave.
9. Extraction buffer
   Final concentrations are: 0.2 M Tris/0.4 M (0.1 M) KCl/0.025 M EGTA/0.035 M $MgCl_2$/1% Triton X-100/0.5% sodium deoxycholate/1 mM spermidine–HCl/5 mM (25 mM) β-mercaptoethanol/0.5 M sucrose
   100 ml H_2O
   50 μl DEPC
   Autoclave. Swirl. While still hot, pour 48 ml into a 250 ml Erlenmeyer flask containing:
   1 g deoxycholate (Sigma)
   51 mg spermidine–HCl (Sigma)
   2 ml Triton X-100
   Shake into solution.
   Add:
   100 ml 2× salts for extraction buffer
   50 ml 2 M sucrose

Keep the solution on ice. Just before the extraction add:
70 μl β-mercaptoethanol (soybean buffer)
350 μl β-mercaptoethanol (tobacco buffer)

10. Sucrose cushion
Final concentrations are: 0.04 M Tris/5 mM EGTA/0.2 M KCl/0.03 M $MgCl_2$/1.8 M sucrose/5 mM (25 mM) β-mercaptoethanol/pH 9
90 ml 2 M sucrose
10 ml 10× salts for sucrose cushion
35 μl (175 μl) β-mercaptoethanol
Cool to 0°C.

11. Resuspension buffer
Final concentrations are: 40 mM Tris/5 mM EGTA/0.2 M KCl/0.03 M $MgCl_2$/5 mM β-mercaptoethanol
45 ml DEPC-treated H_2O
5 ml 10× salts for sucrose cushion and resuspension
17.5 μl β-mercaptoethanol
Cool to 0°C.

12. Sucrose gradients
Make 5 ml of sucrose gradients from the following solutions.

%Sucrose	2M Sucrose (No DEPC) (ml)	10× Salts (ml)	DEPC-treated H_2O (ml)
60	8.8	1	0.2
45	6.6	1	2.4
30	4.4	1	4.6
15	2.2	1	6.8

Construct the gradients as follows: 0.8 ml 60% sucrose, 1.6 ml 45% sucrose, 1.6 ml 30% sucrose and 0.8 ml 15% sucrose. Let the gradients diffuse for 18 h at 4°C.

[a] As the pH gets close to 9.0, a precipitate will form. It will disappear after stirring for a few minutes.
[b] DEPC interferes with UV scanning.
[c] Before using, heat this solution to 40°C with stirring to get the detergents back into solution.

(viii) Spin the supernatant at 10 000 r.p.m. at 4°C for 30 min in a SS34 Sorvall rotor.

(ix) Carefully decant the supernatant into a beaker on ice. Transfer the supernatant to eight 36 ml polycarbonate tubes containing a 5 ml sucrose cushion on ice. Fill the tubes to just above the shoulder.

(x) Spin at 4°C for 16 h at 33 000 r.p.m. in a Ti865 Sorvall rotor.

(xi) Remove the tubes from the rotor and place them at an angle on ice so that the RNA pellets are covered by the sucrose cushion. The pellets will be small and slightly amber.

(xii) Aspirate off the supernatant down to the cushion. Rinse the sides of the tube with cold DEPC-treated H_2O (Section 2.2.2). Aspirate down to the sucrose cushion. Quickly invert the tube so that the pellet is on top and remove the last of the sucrose cushion. Repeat for the remainder of the tubes.

(xiii) Resuspend the pellets in a total of 5 ml of resuspension buffer for 2 h on ice.

Table 3. EDTA-release of polysomal mRNA.

Stock solutions

1. 10× Salts for polysome gradients
   0.4 M Tris
   0.2 M KCl
   0.1 M $MgCl_2$
   Adjust the pH to 8.5 with KOH, treat with DEPC (see Section 2.2.2) and autoclave.
2. 10× Salts for EDTA-release gradients
   0.4 M Tris
   0.2 M KCl
   10 mM EDTA
   Adjust the pH to 8.5 with KOH, treat with DEPC (see Section 2.2.2) and autoclave.
3. 10× Resuspension buffer minus $MgCl_2$
   0.4 M Tris
   2.0 M KCl
   50 mM EGTA
   Adjust the pH to 8.5 with KOH, treat with DEPC (see Section 2.2.2) and autoclave. Add 5 mM BMe to the 1× buffer shortly before use.
4. TES
   10 mM Tris
   0.1 mM EDTA
   0.1% SDS
   Adjust the pH to 7.6, treat with DEPC (see Section 2.2.2) and autoclave.

Procedure

1. Follow steps i–xv of the polysomal RNA isolation procedure (Section 2.5).
2. Layer up to 8 mg on each preparative sucrose gradient. Use 10× polysome gradient salts.

% Sucrose	2 M Sucrose (ml)	10× Salts (ml)	DEPC-treated H_2O (ml) (ml)
32.5	23.8	5	21.2
27	39.4	10	50.6
21	23.0	7.5	44.5
16	11.7	5	33.3
10	7.3	5	37.7

   Construct the gradient as follows: 5.5 ml of 32.5% sucrose, 11.8 ml of 27% sucrose, 7.8 ml of 21% sucrose, 5.0 ml of 16% sucrose and 4.5 ml of 10% sucrose. Let the gradients diffuse for 18 h at room temperature and cool in the 4°C room before use.
3. Spin at 27 000 r.p.m. for 1.25 h in the Sorvall AH627 rotor at 2°C.
4. Under these conditions, the >100S polysomes occupy 63% of the gradient. Fractionate in the 4°C room. Collect the >100S polysomes directly into 1/10 vol. of 3 M sodium acetate and 2 vols of ethanol.
5. Precipitate overnight at −20°C.
6. Pellet the polysomes by spinning the ethanol precipitate at 25 000 r.p.m. for 30 min in the Sorvall AH627 rotor at −10°C. Drain the supernatant and dry the pellet.
7. Resuspend the pellets in 1 ml/pellet of resuspension buffer minus $MgCl_2$ on ice. Determine the RNA recovery.
8. Add EDTA to a final concentration of 0.1 M.
9. Layer up to 3 mg RNA on each preparative sucrose gradient using 10× salts for EDTA gradients instead of 10× salts for polysome gradients. Spin at 27 000 r.p.m. for 8 h in the Sorvall AH627 rotor at 2°C.
10. Under these conditions, the <80S portion occupies 70% of the gradient. Fractionate in the 4°C

room and collect the <80S portion directly into 1/10 vol. of 3 M sodium acetate and 2 vols of ethanol. Precipitate at −20°C overnight.
11. Pellet the <80S mRNA by spinning the ethanol precipitate at 27 000 r.p.m. for 30 min in the Sorvall AH627 rotor at −10°C. Drain the supernatant and dry the pellet.
12. Resuspend the pellets in 0.5 ml of TES/pellet and assay the RNA concentration.
13. Add an equal volume of 1 mg/ml pre-digested (30 min, 37°C, in TES) proteinase K. Incubate at room temperature for 30 min.
14. Add 1/10 vol. of 3 M sodium acetate and 2 vols of ethanol. Store at −20°C.

(xiv) Pellet the remaining insoluble material by spinning at 1000 r.p.m. at 4°C for 10 min in the SS34 Sorvall rotor. Firm up the pellet by briefly increasing the speed to 5000 r.p.m.
(xv) Remove the supernatant and assay the RNA recovery.
(xvi) Assay the polysome profile using sucrose gradients. Layer 80 μg of RNA on the gradient. Spin the gradients at 50 000 r.p.m. at 6°C for 45 min in a Sorvall AH650 rotor. Take the gradients up and down as slowly and as gently as possible (A-slow and reograd on Sorvall centrifuges). Analyse the gradients with a ISCO UV monitor.
(xvii) Remove the ribosomal proteins by adding an equal volume of 1 mg/ml pre-digested proteinase K (pre-digest proteinase K at 37°C for 30 min in 2× TES). Incubate at room temperature for 30 min.
(xviii) Add 1/10 vol. of 3 M sodium acetate (pH 6.0) and 2 vols of 100% ethanol to precipitate the RNA. Store at −20°C.

2.6 Isolation of poly(A^+) RNA

Poly(A^+) RNA can be isolated from total or polysomal RNA by affinity chromatography on oligo-dT–cellulose columns detailed in *Table 4* (12). The RNA is bound to the column in the presence of high salt. The column is then extensively washed with high salt buffer to remove contaminating poly(A^-) RNA (mostly ribosomal RNA). The poly(A^+) RNA is eluted in low ionic strength buffer. We find that two passages through the column are required to remove the contaminating poly(A^-) RNA. This purification method works well for RNA molecules with average to long poly(A) tails (12). *Table 5* shows typical polysomal and poly(A^+) RNA yields from a variety of tobacco organs and from tobacco seeds.

3. NUCLEI ISOLATION AND RUN-OFF TRANSCRIPTION

Regulation of gene expression occurs at many different levels in eukaryotes ranging from processes that initiate transcription to those that control protein turnover. Insight into the mechanisms by which individual genes are regulated requires techniques that can measure these dynamic processes. One such technique, run-off transcription, can be used to measure the relative transcription rates of genes (13–18). It is most often used to measure the relative transcription rates of multiple genes in the same developmental state, or to measure the transcription rates of the same gene in different developmental states, such as in different organs or at different stages of development.

Table 4. Poly(A$^+$) RNA isolation.

Solutions

1. Elution buffer (EB)
   10 mM Tris–HCl (pH 7.6)
   1 mM EDTA
   0.1% SDS
   Treat with DEPC (see Section 2.2.2) and autoclave.
2. Binding buffer (BB)
   0.5 M NaCl
   10 mM Tris–HCl (pH 7.6)
   1 mM EDTA
   0.1% SDS
   Treat with DEPC (see Section 2.2.2) and autoclave.
3. 0.1 M NaOH
   Dissolve the NaOH in DEPC-treated (see Section 2.2.2) and autoclaved water.

Preparation of the columns

1. Place 0.2 g of oligo-dT cellulose over a thin layer of 80 μm glass beads into a glass-jacketed column 1 cm in diameter.
2. Rinse the column with 10 ml of the following solutions: 0.05% DEPC; 0.1 M NaOH, repeat; EB, repeat twice; BB, repeat. This step should be repeated each time the column is re-used.

Isolation of poly(A$^+$) RNA

1. Resuspend the RNA in EB on ice to give a final concentration <0.5 mg/ml.
2. Heat at 68°C for 3 min. Cool quickly on ice.
3. Bring to room temperature and add NaCl to a final concentration of 0.5 M.
4. Pass it over the column at a flow rate of 1 ml/min.
5. Collect the flow through on ice and re-pass it over the column. If the SDS comes out of solution, warm it slightly in a 30°C waterbath.
6. Wash the column with BB until all of the unbound RNA is removed. Assay this by comparing the A_{260} of the flow through with BB.
7. Elute the A$^+$ RNA with EB, collect ten 1 ml aliquots on ice.
8. To remove residual A$^-$ RNA, regenerate the column (see step 2 of Preparation of the columns), heat the poly(A$^+$) RNA to 68°C for 3 min, cool quickly on ice and repeat steps 3–7.
9. Precipitate the RNA by adding 1/10 vol. of 3 M sodium acetate and 2.5 vols of ethanol. Store at −20°C.

To measure relative rates of transcription, nuclei are isolated from the plant material of interest, RNA is elongated from pre-initiated complexes in the presence of radiolabelled nucleotide precursors, and the labelled RNA is hybridized to cloned DNA sequences immobilized on blots. The relative transcription rates of individual genes can be measured by quantitating the hybridization level of the labelled RNA to the cloned DNAs.

The analysis of plant gene regulation by run-off transcription has been optimized by Luthe and Quantrano (17,19). The major modification was the addition of a purification step in which the nuclei are separated from contaminating endogenous RNases on a Percoll gradient.

This section provides detailed protocols for isolating plant nuclei, and synthesizing and isolating nuclear RNA. It has been used successfully to analyse the relative transcription rates of genes expressed in tobacco petals and leaves, tomato fruit, and soybean embryos, leaves, stems and roots (16).

Table 5. Tobacco polysomal RNA and poly(A$^+$) RNA yields.

Source[a]	*Maximum tissue/buffer*[b]	*Yield*[c]	*% Poly(A) RNA*
Leaf (PM)[d]	1:4	1.11 ± 0.47 (5)[e]	0.9 ± 0.2 (5)
Stem	1:5	0.09 ± 0.03 (16)	1.1 ± 0.2 (6)
Root	1:5	0.15 ± 0.05 (13)	0.6 ± 0.1 (5)
Petal	1:5	0.13 ± 0.04 (14)	1.1 ± 0.2 (4)
Ovary	1:8	1.13 ± 0.37 (5)	0.9 ± 0.3 (4)
Anther	1:10	0.70 ± 0.23 (7)	0.9 ± 0.3 (4)
Seed[f]	1:5	0.88 ± 0.34 (22)	0.6 ± 0.2 (22)

[a]Young leaves, 1–4 cm in length, were harvested from 35–50 cm plants. Stems were harvested from 15–20 cm plants cut 2 cm above the soil line. Leaves were excised at the base of the petiole and the shoot apex was removed. Roots were harvested from 35–50 cm plants grown hydroponically. Petals were harvested from fully opened flowers with no sign of senescence. Pistils and anthers were harvested from 1 cm unopened flowers. Filaments were removed by hand-dissection.
[b]We have found that a high tissue to buffer ratio results in lower yields and smaller polysomes. The units are g tissue/ml buffer.
[c]mg polysomal RNA/g tissue.
[d]RNA yields are higher from leaves harvested during peak light hours (noon–2 pm).
[e]The figures in parentheses represent the number of independent RNA isolations.
[f]These seeds were collected 19 days after flowering, the peak of RNA accumulation during tobacco embryogenesis.

3.1 Harvesting plant material

Plant material is harvested and immediately frozen in liquid nitrogen. It can then be stored at −80°C. We have found that fresh tissue and tissue that has been stored at −80°C for more than 1 year result in equally active nuclei.

3.2 Nuclei isolation

In the isolation procedure described in detail below, solutions for which are given in *Table 6*, the cells are lysed in an iso-osmotic buffer containing the non-ionic detergent, Triton X-100. This buffer has been optimized to minimize nuclear damage during extraction (20) and has been used to produce nuclei that are highly active in RNA synthesis (16,17). After lysis, the nuclei are collected by low speed centrifugation and purified on a discontinuous Percoll gradient, a polyvinylpyrrolidone (PVP)-coated silica suspension. Percoll gradients are used to purify plant nuclei instead of the more common sucrose gradients, because they have been shown to be more effective in separating nuclei from endogenous RNases (19). Cellular debris, lipids, chloroplasts and membrane fragments will be found above the 40% Percoll layer. The nuclei will be found either between the 80% Percoll layer and the 2 M sucrose or will pellet depending on their density. Nuclei from soybean embryos at later stages of development, and from tobacco petals, are found between the 80% Percoll layer and the 2 M sucrose. Nuclei from young soybean embryos, leaf, stem, root and tomato fruit pellet, as do the starch granules. The nuclei are washed several times to remove residual Percoll and resuspended in 50% glycerol. They can be

Table 6. Stock solutions for the isolation of nuclei.

1. Honda buffer
   3.3% Ficoll (Sigma 400)
   6.6% Dextran–T40
   33 mM Tris–HCl (pH 8.5)
   6.6 mM $MgCl_2$
   3.3% Triton X-100
   Heat to 45°C to get the Dextran into solution. The final solution will be a bit turbid. Add DEPC to a final concentration of 0.05% (see Section 2.2.2). Autoclave.
2. 200 mM Spermine
   Make this stock in DEPC-treated (Section 2.2.2), autoclaved water. Do not autoclave the spermine solution.
3. Percoll (Pharmacia)
   Autoclave. Store Percoll at 4°C, do not freeze.
4. 3 M Sucrose
   Heat to 75°C to get the sucrose into solution. Autoclave.
5. 10× Percoll gradient buffer
   250 mM Tris–HCl (pH 8.5)
   100 mM $MgCl_2$
   Filter, treat with DEPC (see Section 2.2.2) and autoclave.
6. 2 M Sucrose
   Treat with DEPC (see Section 2.2.2) and autoclave.
7. Nuclei wash buffer
   50 mM Tris–HCl (pH 8.5)
   5 mM $MgCl_2$
   20% Glycerol (v/v)
   Treat with DEPC (see Section 2.2.2) and autoclave.
8. Nuclei resuspension buffer
   50 mM Tris–HCl (pH 8.5)
   5 mM $MgCl_2$
   50% Glycerol (v/v)
   Treat with DEPC (see Section 2.2.2) and autoclave.
9. Honda buffer/DEPC/spermine
   Final concentrations are: 2.5% Ficoll/5.0% Dextran–T40/25 mM Tris (pH 8.5)/5 mM $MgCl_2$/2.5% Triton X-100/0.44 M sucrose/10 mM β-mercaptoethanol/0.04% DEPC/2 mM spermine.
   145 ml Honda buffer
   44 ml 2 M Sucrose
   140 µl β-Mercaptoethanol
   72.5 µl DEPC
   1.89 ml 200 mM Spermine
10. Percoll gradients

% Percoll	Percoll (ml)	3 M Sucrose (ml)	10× Grad buffer (ml)	H_2O (ml)	Volume (ml)
40	8	3	2	7	20
60	12	3	2	3	20
76	16	3	2	0	21
0	0	13	2	6	21

Put the solutions in ice. It is easier to layer the solutions when they are cold. The gradients are formed in 30 ml Corex tubes. The steps are layered from the bottom up. Each layer is 4.5 ml. Let the gradients stand at 4°C for at least 30 min before they are used.

stored at −80°C for at least a year. General guidelines for RNA isolation (Section 2.2) should be followed. The glassware and solutions must be RNase-free. Carry out steps i–v in the coldroom.

(i) Grind the tissue to a fine powder in liquid nitrogen in a Waring blender.
(ii) Transfer the powder and liquid nitrogen to an omni-mixer cup and allow the liquid nitrogen to evaporate. When the liquid nitrogen is completely evaporated, the tissue will be lighter in colour and will not clump. It is important that the liquid nitrogen evaporates so that the buffer will not freeze. However, it is even more important that the tissue does not thaw.
(iii) Add a 10× excess (v/w) of Honda buffer/DEPC/spermine to the powder (190 ml of Honda/19 g of tissue).
(iv) Homogenize in the omni-mixer on ice at top speed for 30 sec. Repeat twice.
(v) Filter the homogenate through 60–80 μm mesh Nitex [DEPC treat (see Section 2.2.2) and autoclave before use, Small Parts Inc., Miami, FL].
(vi) Spin the homogenate at 5000 r.p.m. for 5 min using the Sorvall JA20 or SS34 rotor at 4°C. This will pellet the nuclei.
(vii) Remove the supernatant by vacuum aspiration.
(viii) Using a Pasteur pipette, resuspend the nuclei in 1 ml of Honda buffer/sucrose/β-mercaptoethanol (no DEPC or spermine) per pellet on ice. If the pellet contains a lot of starch, it will be very hard and difficult to resuspend. In this case, disperse the pellet with a glass rod before resuspending with a Pasteur pipette. Pool the nuclei and bring the volume up to 20 ml (total) with Honda buffer/sucrose/β-mercaptoethanol. If the nuclei are contaminated with other compounds, resuspend and repeat the spin.
(ix) Gently layer about 5–5.5 ml of nuclei on each of four Percoll gradients.
(x) Spin the Percoll gradients in a Sorvall HB-4 swinging bucket rotor for 30 min at 5000 r.p.m. at 4°C.
(xi) Remove the upper layers with a vacuum aspirator. If the nuclei pellet, leave about 2 ml in the tube. Resuspend and remove the nuclei with a Pasteur pipette. When isolating nuclei from developmental stages or from organs where starch, polysaccharides or protein bodies are a major problem, a second round of Percoll gradients are carried out.
(xii) Pool the nuclei. Add 15–20 ml of Honda buffer/sucrose/β-mercaptoethanol and mix with a Pasteur pipette until there are no aggregates.
(xiii) Spin down the nuclei in a Sorvall HB-4 rotor at 5000 r.p.m., for 5 min at 4°C. Remove the supernatant by vacuum aspiration.
(xiv) Resuspend the nuclei in 2 ml of Honda buffer/sucrose/β-mercaptoethanol on ice with a Pasteur pipette. Bring the volume up to 20 ml with the same solution. Repeat step xiii.
(xv) Add 2 ml of nuclei wash buffer/10 mM β-mercaptoethanol to the pellet. Resuspend the pellet with a Pasteur pipette on ice. Add 18 ml of nuclei resuspension buffer/10 mM β-mercaptoethanol and mix. Repeat step xiii.
(xvi) Resuspend the nuclei in a minimal volume of nuclei resuspension

buffer/10 mM β-mercaptoethanol. We have resuspended the nuclei from 19 g of tissue in 400 µl.

(xvii) Put into a pre-chilled box and store at −80°C. The nuclei appear to be stable at −80°C for a long period of time (>1 year). This is assayed by their ability to incorporate UTP and by the analysis of their transcription products.

3.3 RNA labelling

A detailed procedure for RNA synthesis in isolated nuclei is provided in *Table 7*. RNA is synthesized in 100 mM $(NH_4)_2SO_4$ and 4 mM $MgCl_2$,

Table 7. *In vitro* transcription.

Do not DEPC treat any of these solutions.

1. 10× Salts solution
   1 M $(NH_4)_2SO_4$ (2 M stock—filtered, autoclaved.)
   40 mM $MgCl_2$ (1 M stock—filtered, autoclaved.)
   3 µM Phosphocreatine (3 mM stock—made in autoclaved H_2O, pH 7.0)
2. 5 mM ATP, GTP, CTP in autoclaved H_2O. Adjust the pH to 7.0 with NaOH.
3. 20 µg/ml α-Amanitin in autoclaved H_2O.
4. 2.5 mg/ml Creatine phosphokinase in autoclaved H_2O.
5. Stop buffer
   0.2% SDS
   5% Na_4PPi
   2 mM UTP
   0.5 M Phosphate buffer

Test synthesis

1. Thaw nuclei on ice and mix gently to disperse the nuclei.
2. Add (in the following order) and incubate for 20 min at 30°C.

Nuclei[a]	10 µl
10× salts	2 µl
H_2O[b]	2 µl
0.25 mg/ml Creatine phosphokinase (1:10 dilution of stock)	2 µl
ATP, GTP, CTP stock	2 µl
[^{32}P]UTP (3000 Ci/mM, 10 mCi/ml)	2 µl

3. Stop the reaction by adding 100 µl of Stop buffer. Use Stop buffer only when testing the nuclei. For the preparative synthesis, go directly to step 1 of the RNA isolation (*Table 8*).
4. Vortex the reaction at a high speed for a few seconds to decrease the viscosity of the reaction so that an accurate volume can be sampled.
5. Assay incorporated counts by TCA precipitation or DE81 filter analysis.

Preparative synthesis

1. Scale up and carry out preparative synthesis at 30°C for 20 min. Follow steps 1 and 2 of test synthesis and then continue with step 1 of the RNA isolation (*Table 8*).

[a] Use a yellow pipetman tip with the end cut off to transfer the nuclei.
[b] 20 µg/ml α-Amanitin can be used in a separate reaction instead of H_2O (final concentration 2 µg/ml α-amanitin) to determine what percent of the synthesized RNA is made from polymerase II.

monovalent and divalent cation concentrations in which RNA polymerase has maximum activity (17). The concentration of unlabelled nucleotides is 500 μM. The reaction is incubated at 30°C for 20 min. We have observed that the rate of synthesis is linear during this time.

Synthesis rates will vary depending on the source of the nuclei. Because we have observed as much as a 10-fold variability in the amount of RNA synthesized from nuclei from various sources, we suggest that a test reaction be done first. After this the reaction can be scaled up by increasing the reaction volume and number of nuclei, or by increasing the [^{32}P]UTP in the reaction. We synthesize approximately 1×10^8 d.p.m. of incorporated counts and use at least 1×10^7 d.p.m. per blot (75–150 cm^2).

To determine how much of the synthesis is due to RNA polymerase II activity, a separate aliquot of nuclei is incubated in the presence of 2 μg/ml α-amanitin (*Table 7*). At this concentration, most of the polymerase II activity is inhibited while polymerases I and III are unaffected. We find that 40–80% of the RNA synthesis is inhibited at this α-amanitin concentration.

3.4 RNA isolation

After the RNA has been synthesized, the DNA and proteins are removed by enzymatic digestion and phenol extraction (*Table 8*). The RNA is purified from other cellular components by centrifugation through a CsCl cushion. Unincorporated nucleotides and CsCl are removed by chromatography on a 25 ml column of Sephadex G-150. The RNA is then separated from contaminating polysaccharides by extraction with hexadecylpyridinium chloride (CTAB) (21) (see *Table 9*). This purification step reduces non-specific background binding when the RNA is used as a hybridization probe. The rationale behind this extraction is that the quaternary ammonium cation in CTAB forms a complex with the anionic nucleic acid under low salt conditions. In the two-phase water–butanol system the complex partitions to the alcohol phase and the polysaccharides and other contaminants remain behind in the water phase. When the salt concentration is raised, the complex dissociates and the nucleic acid can be recovered in the water layer. Chloroform is then used to remove residual CTAB.

3.5 Hybridization

The labelled RNA can be used to determine the relative rates of gene transcription by one of the following two methods. The RNA can be hybridized to cloned DNA sequences dotted onto a nitrocellulose filter (15) or to DNA separated by electrophoresis on an agarose gel and blotted onto nitrocellulose (18). The resulting hybridization can be quantitated either by autoradiography followed by densitometric analysis or by cutting out the dots and counting them in a liquid scintillation counter.

Table 8. RNA isolation.

Stock solutions

1. 10× TES
   100 mM Tris–HCl (pH 7.6)
   50 mM Na_2EDTA
   10% SDS
   Make the SDS in sterile, autoclaved water and add to Tris and EDTA that have been autoclaved.
2. TESar
   10 mM Tris–HCl (pH 7.6)
   1 mM Na_2EDTA
   1% Sodium lauroylsarcosinate
   Filter and autoclave.
3. Phenol
   Double-distilled phenol is equilibrated with 50 mM Tris–HCl (pH 8.0), 0.1 mM EDTA.
4. Phenol/Sevag
   Equal volumes of phenol and Sevag are mixed together. Sevag is chloroform and isoamyl alcohol (24:1).

Procedure

1. Add RNase-free DNase to a final concentration of 20 μg/ml. Incubate for 10 min at 30°C.
2. Add 1/10 vol. of 10× TES.
3. Add pre-digested Proteinase K (pre-digest, 30 min, 37°C in TESar) to a final concentration of 100 μg/ml. Incubate for 30 min at 42°C.
4. Add 50 μg of tRNA. The tRNA should be phenol/Sevag and Sevag extracted and ethanol precipitated before use.
5. Extract with an equal volume of phenol/Sevag at room temperature. Re-extract with a small volume of TES. Repeat until no interface can be detected after spinning.
6. Add 1/10 vol. of 3 M sodium acetate (pH 6.0) and 2.5 vols of ethanol. Precipate overnight at −20°C.
7. Pellet the RNA for 30 min at 4°C in the microfuge and dry.
8. Resuspend the RNA in 400 μl of TESar on ice.
9. Add 400 μl of 1 g/ml CsCl in TESar (filtered and autoclaved).
10. Put 1.2 ml of 5.7 M CsCl/TESar into a 5 ml Sorvall AH650 tube. Gently layer the 0.8 ml of RNA/CsCl onto the 5.7 M CsCl cushion. Fill the tube with 0.5 g/ml CsCl/TESar.
11. Spin at 35 000 r.p.m. for 46 h at 20°C in a AH650 Sorvall rotor.
12. Carefully remove all of the gradient except for the last 0.2 ml. Assay this aliquot for incorporated counts. If that fraction has >20% of the total incorporated counts in the pellet, add that aliquot to the resuspended pellet.
13. Resuspend the pellet in 200 μl of TESar. Rinse the tube with an additional 200 μl of TESar. Pool the aliquots.
14. Run the RNA over a 25 ml Sephadex G-150 column equilibrated against TESar[a].
15. Collect the RNA and add 1/10 vol. of 3 M sodium acetate (pH 6.0) and 2.5 vols of ethanol.
16. Precipitate overnight at −20°C.

[a]We use a column that is 1.2 cm in diameter and 26 cm long. We collect 35 drop fractions and the volume of the pooled fractions is ~7 ml.

Table 9. CTAB purification of RNA.

Solutions

CTAB/butanol and CTAB/aqueous

(i) In a separatory funnel, shake 75 ml of 1-butanol and 75 ml of H_2O. Allow the phases to separate. Collect the phases.

(ii) Add 1.84 g of CTAB to 50 ml of the butanol saturated with water. Add 50 ml of water saturated with butanol. Shake in a separatory funnel. Allow the phases to separate overnight.

(iii) Butanol/CTAB (Bu/CTAB) is the upper layer and aqueous/CTAB (Aq/CTAB) is the lower layer. Store separately.

Procedure

1. Pellet the RNA by spinning at 22 000 r.p.m. for 1 h at 4°C and dry.
2. Resuspend the pellet in 200 µl of TESar (*Table 8*).
3. Add 200 µl of Aq/CTAB and 200 µl of Bu/CTAB.
4. Vortex for at least 2 min.
5. Separate the phases by spinning in a microfuge for 2 min.
6. Transfer the upper (butanol) phase to a microfuge tube. This phase will contain the nucleic acids.
7. Re-extract the aqueous phase with 200 µl of Bu/CTAB. Pool the butanol layers.
8. Add 150 µl of 0.2 M NaCl. Vortex for 30 sec. Separate the phases by spinning in a microfuge for 2 min.
9. Transfer the lower (aqueous) phase to a microfuge tube. This now contains the RNA.
10. Re-extract the butanol phase with 150 µl of 0.2 M NaCl. Pool the aqueous layers.
11. Add 300 µl of chloroform, dropwise, to the pooled aqueous phases. Incubate on ice for 15 min.
12. Separate the phases by spinning in the microfuge for 2 sec.
13. Remove the lower (chloroform) phase which now contains the CTAB.
14. Ethanol precipitate the aqueous layer by adding 1/10 vol. of 3 M sodium acetate (pH 6.0) and 2.5 vols of ethanol.
15. Precipitate overnight at −20°C.

4. SYNTHESIS OF SINGLE-STRANDED RNA PROBES FOR RNA BLOT ANALYSIS

4.1 Introduction

Single-stranded, high specific activity RNA sequences are very efficient hybridization probes. They can be used in a variety of analyses including *in situ* hybridization (Section 6), blot analysis (Section 4) and RNA titration analysis (Section 5). RNA probes have been shown to be as much as ten times more sensitive than nick translated probes of the same specific activity in blot analysis (22) and *in situ* hybridization (23). This section will describe in detail synthesis of RNA probes, as well as provide suggestions for their use. A more detailed discussion of the uses of these probes can be found in other sources (22,24).

4.2 Template preparation

The synthesis of single-stranded RNA probes was made possible by the construction of vectors containing bacteriophage promoters (22), the most common of which are SP6, T7 and T4, and the commercial availability of their respective RNA polymerases. Although many of these vectors are now available, we recommend the use of a vector system similiar to pGEM Blue

(Promega Biotec). This vector contains both the SP6 and T7 RNA polymerase promoters flanking a multiple cloning region. After insertion of the cloned DNA into the multiple cloning site, both the coding and non-coding strands can be synthesized from a single recombinant clone using the appropriate polymerase. In addition, it also contains the lac α-peptide which complements the lacZ M15 gene to produce β-galactosidase and can be used to identify clones containing inserts by colour selection.

We generally use DNA that has been purified on CsCl gradients for *in vitro* transcription templates. However, others have shown that intact RNA can be synthesized using DNA templates prepared by mini-lysate procedures (25).

After isolation of the template, the clone is linearized downstream of the insert by digestion with the appropriate enzyme (*Table 10*). The synthesis of 'run-off' transcripts from truncated templates eliminates labelled vector from subsequent hybridization reactions. This increases the sensitivity of the blot by lowering non-specific background binding. Restriction enzymes that produce 3′ overhangs should be avoided (25). There is evidence that non-specific initiation can occur at these sites giving rise to RNA from the opposite strand and the vector. If a restriction enzyme that produces a 3′ overhang must be used, the end should be converted to blunt form using T4 DNA polymerase or the Klenow fragment of DNA polymerase I. After linearization, the DNA is phenol extracted and precipitated.

4.3 *In vitro* transcription

In this system, transcription begins at the bacteriophage promoter, proceeds through the cloned insert and terminates when the enzyme reaches the end of the template. The detailed transcription procedure, provided in *Table 11*, is a modification of the procedures of Melton *et al.* (22). They have determined optimal transcription reaction conditions, and several aspects of their work will be discussed below.

4.3.1 *Nucleotide concentration*

Measurements of the amount and length of the transcribed RNA have been made at various ribonucleotide concentrations. All four nucleotides saturate at 250 μM. However, nucleotide concentrations of this magnitude are not practical

Table 10. Preparation of template DNA.

1. Digest the DNA with the appropriate restriction enzyme.
2. Add 1 vol. of 1 mg/ml pre-digested [30 min, 37°C, in 20 mM Tris–HCl (pH 7.0), 0.2 mM EDTA and 2% sodium lauroylsarcosinate] Proteinase K. Incubate at 37°C for 30 min.
3. Extract with an equal volume of phenol/Sevag (*Table 8*). Extract with an equal volume of Sevag. Double-distilled phenol is equilibrated with 50 mM Tris–HCl (pH 8.0), 0.1 mM EDTA.
4. Add ⅒ vol. of 3 M sodium acetate (pH 6.0) and 2 vols of ethanol. Precipitate overnight at −20°C.
5. Spin down the precipitate in a microfuge at 4°C. Wash the pellet with 70% ethanol and spin. Dry the pellet.
6. Resuspend the DNA in TE (10 mM Tris, pH 7.0, 0.1 mM EDTA) at 1 μg/μl.

for synthesizing high specific activity probes. We routinely use a concentration of 250–500 μM for the three unlabelled nucleotides and 12–25 μM for the labelled nucleotide. At these concentrations, it is still possible to incorporate 50–80% of the labelled nucleotides and to get a large fraction of full length probe.

4.3.2 *Time course*

The reaction rate is constant for about 1 h and drops off slowly after that.

4.3.3 *Temperature optimum*

A temperature curve for RNA synthesis shows a sharp temperature optimum at 40°C. Synthesis occurs at approximately 10% of this rate at 30°C or 50°C.

Table 11. RNA transcription.

Solutions

1. 5× Transcription buffer
   200 mM Tris–HCl (pH 7.5)
   30 mM $MgCl_2$
   10 mM Spermidine
   50 mM NaCl
   DEPC treat (Section 2.2.2) and autoclave all the components except the spermidine. Filter sterilize the spermidine stock.
2. 100 mM Dithiothreitol (DTT) in DEPC-treated H_2O (Section 2.2.2).
3. 10 mM NTP stocks (10 mM ATP, 10 mM UTP, 10 mM GTP) in DEPC-treated H_2O (Section 2.2.2) and adjust the pH to 7.0.

Procedure

1. Combine the following[a]:

	Final concentration
5× Transcription buffer	1×
100 mM DTT	10 mM
RNasin (25 U/μl)	1 U/μl
10 mM ATP, UTP, GTP	250–500 μM each
Template	0.1 μg/μl
[^{32}P]CTP[b], (400 Ci/mmol, 10 mCi/ml)	12–25 μM
SP6 or T7 Polymerase (10 U/μl)	0.2 U/μl

   Final volume: 50 μl. Incubate for 45 min at 40°C.
2. Add RNase-free DNase (Promega Biotec) to a final concentration of 1 U/μg DNA. Incubate for 15 min at 37°C.
3. Add 10 μg of yeast tRNA carrier.
4. Add 1 vol. of 1 mg/ml pre-digested [30 min, 37°C, in 20 mM Tris–HCl (pH 7.0), 0.2 mM EDTA, 2% sodium lauroylsarcosinate] Proteinase K. Incubate for 30 min at room temperature.
5. Extract with an equal volume of phenol/Sevag (*Table 8*). Extract with an equal volume of Sevag.
6. Pass the RNA over a Sephadex G-50 column to remove unincorporated nucleotides[c].
7. Add 1/10 vol. of 3 M sodium acetate and 2.5 vols of ethanol. Precipitate overnight at −20°C.

[a] Combine the components in the order shown. The mixture should be kept at room temperature. Because of the spermidine, the DNA can sometimes precipitate if incubated on ice.
[b] The reaction can be run in the absence of unlabelled CTP if maximum specific activity is desired. For a 50 μl reaction, 250 μCi of 400 Ci/mmol [^{32}P]CTP is needed. This is ~12 μM. The yield of full length transcripts is reduced as the concentration of the nucleotides falls below 12 μM.
[c] This step is not required for preparation of *in situ* probes.

4.3.4 *Length of RNA transcripts*

Full length transcripts are made from virtually any size DNA template, although varying amounts of transcripts shorter than full length are observed. In addition, these RNA polymerases are able to transcribe continuous stretches of poly(C), poly(G) and poly(A) of about 20 bases without termination.

4.3.5 *Specificity*

These phage polymerases are totally specific for their own promoters and will not initiate transcription on other prokaryotic or eukaryotic promoters.

4.4 Hybridization

Many procedures have been published for RNA blot analysis using RNA probes (22,26,27). Two of these are described in *Table 12*. In addition, procedures developed for double-stranded DNA probes can also be used for single-stranded RNA probes with the following modifications.

(i) Hybridization and wash criteria must be raised. RNA–RNA hybrids have a higher thermal stability than DNA–RNA hybrids. The melting temperature (T_m) in solution of a RNA–RNA hybrid of average GC content in 50% formamide and 0.3 M NaCl is approximately 80°C. Optimum hybridization rates occur at $T_m - 25$. Therefore, RNA blot hybridizations using RNA probes are generally incubated at 55–60°C. Some researchers have even found higher temperatures (65–70°C) preferable because they result in less non-specific background binding (26). Wash temperatures should also be raised for the same reason.

(ii) Hybridization buffers must be prepared RNase-free. This includes DEPC-treating the buffers and glassware, and using RNase-free methods for experimental manipulations (see RNA extraction, Section 2.2).

(iii) Yeast RNA should be included as an RNA competitor in the pre-hybridization and hybridization mix.

(iv) An RNase A wash can be included to further lower the non-specific background binding of probe (27).

5. RNA TITRATION ANALYSIS

5.1 Introduction

RNA titration analysis is the most accurate and the most sensitive method to measure the absolute number of copies of a particular RNA transcript in an organism, organ, tissue or cell. Briefly, excess labelled RNA homologous to the sequence of interest (tracer) is hybridized to various amounts of cellular RNA. After the hybridizations have reached completion, unhybridized tracer is digested with RNase and the tracer–cellular RNA hybrids are assayed. Under tracer-excess conditions, a plot of the amount of hybridized tracer versus the amount of cellular RNA for each reaction yields a straight line, the slope of which represents the percent of the cellular RNA that is homologous to the

Table 12. Hybridization conditions.

METHOD 1

Hybridization solution

50% Formamide
5× SSC (0.75 M NaCl, 75 mM sodium citrate, pH 7.0)
0.1 M Sodium phosphate (pH 6.5)
1× Denhardt's (10× Denhardt's in the pre-hybridization solution) 10× Denhardt's is 0.2% bovine serum albumin (BSA), 0.2% Ficoll and 0.2% PVP
0.1% SDS
250 μg/ml tRNA
100 μg/ml Sheared single-stranded salmon sperm DNA
10% Dextran sulphate (no Dextran sulphate in the pre-hybridization solution)

Procedure

1. Pre-hybridize the filter in hybridization buffer without Dextran sulphate and with 10× Denhardt's overnight at 55°C.
2. Denature the probe at 90°C for 10 min.
3. Hybridize with 3×10^6 c.p.m. of probe per ml. The total volume is 0.3 ml of hybridization buffer per cm^2 of blot. Incubate for 20 h at 55°C.
4. Wash with 50% formamide, 5× SSC, 0.1 M sodium phosphate and 0.1% SDS for 30 min at 55°C. Repeat five times.

METHOD 2

Hybridization solution

50% Formamide
0.1 M Pipes (pH 6.8)
0.5 M NaCl
0.2% SDS
10× Denhardt's (0.2% BSA, 0.2% Ficoll, 0.2% PVP)
250 μg/ml Yeast RNA (Sigma type III)

Procedure

1. Pre-hybridize the filter in hybridization buffer for >3 h at 68°C.
2. Hybridize with 1×10^6 d.p.m. of probe per ml of hybridization solution. Use 0.1 ml of hybridization solution per cm^2 of blot. Hybridize for 20 h at 68°C*.
3. Wash briefly with 2× SSC at room temperature. Repeat. Wash with 0.2× SSC, 0.01% SDS at 68°C for 20 min. Repeat three times.

*Nytran or a similar nylon membrane will withstand a 68°C incubation better than nitrocellulose.

tracer. If the number of cells expressing the gene and the total number of RNA molecules in those cells are known, the absolute number of molecules of a given sequence per cell can be calculated. The details of this technique are described in *Table 13* (28,29).

Correct interpretation of such data requires that the following criteria be met.

(i) The tracer must be in excess (Section 5.3.1) and the reaction must go to completion (Section 5.3.2). It is only under these conditions that the percent of the tracer in hybrid is proportional to the amount of input cellular RNA and that a linear positive slope will be generated. As

Table 13. RNA titration analysis.

Solutions

1. 5× Hybridization mix
   2 M NaCl
   125 mM Pipes
   1 mM EDTA
   Adjust the pH to 6.8, filter through 45 μm Millipore filter, DEPC treat (Section 2.2.2) and autoclave.
2. SET
   0.375 M NaCl
   75 mM Tris
   5 mM EDTA
   Adjust the pH to 8.0, filter through 45 μm Millipore filter and autoclave.

Probe synthesis

1. Synthesize a ^{32}P-labelled RNA tracer with a specific activity of 6×10^8 d.p.m./μg using the procedures detailed in Section 4, *Table 10* (steps 1–6) and *Table 11* (steps 1–7).

Hybridization

1. Mix the appropriate amounts of tracer (generally 50–200 pg) and cellular RNA.
2. Add yeast total RNA to a final RNA content of 100 μg. [The yeast RNA should be digested for 30 min at 37°C with 1 mg/ml proteinase K, extracted with an equal volume of double-distilled, equilibrated (50 mM Tris, pH 8.0, 0.1 mM EDTA) phenol:Sevag (*Table 8*) and an equal volume of Sevag, ethanol precipitated and resuspended in DEPC-treated water (Section 2.2.2) before use.]
3. Add 1/10 vol. of 2 M ammonium acetate and 2 vols of ethanol. Precipitate overnight at −20°C.
4. Spin down the precipitate in a microfuge at 4°C. Wash the pellet with 70% ethanol and dry. Resuspend in 6 μl of DEPC-treated water.
5. Add 10 μl of 100% de-ionized formamide.
6. Add 4 μl of 5× hybridization mix.
7. Cover the reaction with 50 μl of mineral oil[a].
8. Heat to 85°C for 5 min.
9. Hybridize at 50°C[b] for the calculated time (see Section 5.3.2).

Hybridization assay

1. Stop the hybridization by placing the reaction on ice.
2. Dilute the samples in 280 μl of SET.
3. Add sufficient RNase A and RNase T1 to get complete digestion of single-stranded RNA[c]. Incubate at 37°C for 1 h.
4. Assay undigested counts using TCA precipitation of DE81 filters.

[a] An alternative method for this is to seal the reaction in DEPC-treated, silanized and baked capillary tubes.
[b] 50°C is the optimum hybridization temperature for 500–1000 nt probes of average GC content.
[c] The activity of each RNase lot should be titrated. We find that 100 μg of RNase A and 225 units of RNase T1 is sufficient to digest 100 μg of RNA to completion.

saturation of the tracer molecules is approached, the slope of the curve describing tracer hybridized versus input of cellular RNA approaches zero.

(ii) The precise specific activity of the tracer must be known. This is possible with the *in vitro* transcription systems (Section 4.3) in which the specific activity of the tracer is dependent only on the specific activity of the nucleotide precursors.

(iii) The tracer's specificity at the hybridization criteria and the length of the tracer which will hybridize to the RNA transcript must be known.

RNA titration analysis is very sensitive. If the background level of RNase-resistant tracer is low (<0.5% of the total counts), 0.1–0.3 pg of a specific RNA transcript can be measured (29). We have observed that some tracers contain a larger percent of RNase-resistant nucleotides due to foldback regions. In these instances, a preliminary analysis of different restriction fragments in the tracer may be necessary in order to identify and delete these regions.

5.2 **Probe synthesis**

The tracer must be uniformly labelled and single-stranded. It can be either a single-stranded RNA synthesized using the *in vitro* transcription technique outlined in Section 4 (22), or a single-stranded DNA made from an M13 vector (30). We recommend use of a single-stranded RNA tracer for the following reasons.

(i) The tracer is completely single-stranded. Complementary sequences in the template are removed by DNase digestion.

(ii) The hybridization can be done at higher stringency since RNA–RNA duplexes are more stable than DNA–RNA duplexes. This tends to decrease non-specific background signals.

(iii) Differentiation of unhybridized single-stranded tracer and hybridized double-stranded tracer is highly reproducible because RNA–RNA duplexes are completely resistant to RNase in moderate to high ionic strength buffers (28).

5.3 **Hybridization reaction**

5.3.1 *Tracer/cellular RNA ratio*

The labelled tracer RNA must be present at greater than 5-fold sequence excess. An estimate of the concentration of the RNA sequence being analysed must be made. This is most easily done by an initial RNA blot analysis where the intensity of the signal for the specific RNA is compared to the signal obtained from hybridization of a probe to an RNA of known prevalence. If this preliminary analysis cannot be done, hybridizations should be carried out with a wide range of cellular RNA concentrations to determine a range in which the amount of tracer in duplex is proportional to the input of cellular RNA.

5.3.2 *Hybridization time*

Since the concentration of unreacted tracer does not change significantly during the hybridization, titration reactions follow pseudo-first-order kinetics. We suggest that each reaction be hybridized to $10\times\ R_0T_{1/2}$, that is 10 times the length of time required for 50% of the hybrids to form. $R_0T_{1/2}$ can be calculated from equation 1.

$$R_0T_{1/2} = \ln 2/K_t \quad (1)$$

R_0 is the concentration of the tracer in moles of nucleotides per litre, T is the incubation time in seconds and K_t is the rate constant. The rate constant for any single-stranded RNA tracer can be calculated by assuming kinetics similar to single-stranded DNA tracers and using the following standard. A sequence with a complexity of 5400 nt hybridizes with a rate constant of 169 M^{-1} sec^{-1} under standard conditions (T_m − 25, 0.18 M Na^+, fragment length = 400–500 nt) (31). This standard can be inserted into equation 2 to determine the rate constant, K_t, for the tracer.

$$K_t = K_s \times C_s/C_t \quad (2)$$

$$K_t = 169\ M^{-1}\ sec^{-1} \times 5400\ nt/C_t$$

C_t equals the complexity of the tracer. In titration experiments where the tracer consists of multiple copies of a single RNA sequence, the complexity is equal to the number of nucleotides in the sequence.

The RNA titration hybridization is not done under standard conditions. These factors should be used in arriving at a final rate constant.

(i) Under aqueous conditions, 0.4 M NaCl increases the hybridization rate 4-fold (32).
(ii) 50% formamide retards the rate 2-fold (33).
(iii) The hybridization rate changes as the square root of the probe length (34).

Due to the low complexity of the tracer, $10\times$ $R_0T_{1/2}$ can generally be reached in 24–48 h.

5.4 Analysis of the data

5.4.1 *Amount of hybridized tracer*

$$\text{Tracer hybridized} = (\text{c.p.m.}^h - \text{c.p.m.}^b)/(\text{sp. act.} \times C) \quad (3)$$

The amount of tracer hybridized in each reaction can be calculated using equation 3. C.p.m.^{h} is the counts per minute resistant to RNase digestion after incubation of the tracer with the cellular RNA. C.p.m.^{b} is the counts per minute resistant to RNase digestion after incubation of the tracer alone. Sp. act. is the specific activity of the tracer and C is the counting efficiency of the isotope.

5.4.2 *Fraction of the cellular RNA homologous to the tracer*

$$F = S \times L_r/L_t \quad (4)$$

The fraction of the cellular RNA that is homologous to the tracer can be calculated using equation 4. S is the slope of the line generated by plotting the amount of hybridized tracer (Section 5.4.1) versus the amount of cellular RNA in each reaction. L_r is the length of the RNA sequence being assayed and L_t is

the length of the tracer in hybrid. Obtaining a non-linear plot or a slope of zero suggests that the tracer RNA was not in sufficient excess and that the hybridizations should be redone with a higher input of tracer molecules.

5.4.3 *Number of molecules of homologous RNA per cell*

$$\text{molecules/cell} = (F \times R \times 6 \times 10^{23})/(E \times 339 \times L_r) \quad (5)$$

The absolute number of molecules per cell of RNA homologous to the tracer can be calculated using equation 5. F is the fraction of the cellular RNA that is homologous to the tracer (Section 5.4.2). R is the amount of RNA per cell (see below). 6×10^{23} is the number of nucleotides per mole. E is the fraction of the total number of cells assayed that express the gene (see below). The average molecular weight of a ribonucleotide is 339 and L_r is the length in nucleotides of the RNA sequence being assayed. In order to calculate the number of molecules of a particular sequence per cell, it is necessary to know the total RNA content per cell. This is most easily done by determining the RNA content of a mass of cells using the phloroglucinol reaction (35) and determining the number of cells by measuring the amount of DNA using the diphenylamine procedure (36). From these measurements, the average RNA content per cell can be calculated. It is important to remember that this is the average and that different cell types may have different RNA contents. A second value required for this calculation is the percent of cells included in the assay that are expressing the gene. This can be measured by *in situ* hybridization techniques discussed in Section 6.

6. IN SITU HYBRIDIZATION

6.1 Introduction

In situ hybridization is used to analyse spatial patterns of RNA accumulation at the cellular and subcellular levels. In this technique, labelled RNA probes are hybridized *in situ* to homologous RNA sequences in cytological preparations. Use of an autoradiographic emulsion allows the direct identification of cells that contain target RNA sequences.

In situ hybridization has already proved to be a powerful technique for analysing gene expression in animals, particularly during embryogenesis. Recently this technique has been modified for plant systems where we believe it will have similar utility. It can be used to assay gene expression at stages of plant development when standard molecular techniques cannot be used, such as, early stages of embryogenesis or organogenesis when the embryo or organ is very small and cannot be dissected from other structures. This hybridization technique can also be used to identify RNA localization patterns when there are no available methods for biochemical isolation of individual cell types.

This section will discuss various aspects of *in situ* hybridization and provide procedures that have been used successfully in our laboratory and others to analyse RNA localization patterns in all tobacco organs, *Lemna* roots and fronds, maize leaves and tobacco (37), soybean and *Brassica* embryos. Other

techniques such as that described in Chapter 5 have been published, which use alternative fixatives, sectioning methods and pre-hybridization treatments (38–40). Depending upon the plant, the organ, and perhaps the developmental stage to be analysed, one procedure may be superior to others.

RNase-free methods (see RNA extraction, Section 2.2) should be used for all experimental manipulations until the post-hybridization washes.

6.2 Fixation, dehydration, clearing and embedding

Optimal fixation of plant material for *in situ* hybridization has two goals. The first is good preservation of tissue morphology and second is retention of RNA molecules at their *in vivo* positions. This must be accomplished without overfixation of the tissue which limits the accessibility of the target RNA to the probe. There are a considerable number of standard fixation methods for plant tissue. They can be grouped into two classes, those that fix by precipitating molecules and those that fix by crosslinking, such as the aldehydes. Early work has conclusively shown that crosslinking fixatives are more effective at retaining RNA molecules during *in situ* hybridization (41,42). Two fixation procedures have been provided in *Table 14*. The glutaraldehyde fixation method has been used successfully by this laboratory and others for fixation of tobacco leaves, stems, roots, petals, ovaries and anthers, *Lemna* roots and fronds, maize leaves and soybean roots and embryos. This fixation procedure was not successful for fixing late stage embryos in tobacco seeds (37). For this plant material, we found that the more standard plant fixative FAA (formalin, alcohol, acetic acid) was superior (43). This is probably due to better penetration of the tobacco seed coat by the smaller formalin molecules. Direct comparisons of *in situ* signals resulting from hybridization of root tips fixed with FAA or glutaraldehyde show that there is no detectable difference in the hybridization efficiency.

We have used ethanol and xylene for dehydration and clearing steps. Tertiary butyl alcohol is recommended by some sources because it is a gentler clearing agent and results in tissues that are less hardened. Tertiary butyl alcohol is compatible with this technique. It is important to remember that regardless of which chemicals are used, changes in concentration should be gradual. Drastic changes cause shrinking of cytoplasm and cellular distortion.

Plant organs that contain cells with large vacuoles should be dehydrated under vacuum to facilitate infiltration of the solutions, otherwise the tissue will not be properly embedded. All changes in pressure should be gradual to avoid tearing.

Finally, all tissues and organs are different in their biochemical composition. Fixation, dehydration, clearing and embedding techniques may have to be optimized. In changing these procedures it is important to remember that some of these steps are interrelated. For example, the extent of fixation as well as the choice of a fixative will have an effect on the optimum extent of protein digestion during pre-hybridization and the optimum probe length for hybridization. Changes in any portion of the technique may have to be coordinated with changes at other steps.

Table 14. Fixation, dehydration, clearing and embedding.

Glutaraldehyde fixation

1. Cut the tissue and immediately place it in a vial containing 10 ml of 1% glutaraldehyde in 0.05 M sodium cacodylate trihydrate buffer[1] (pH 7.0) (Ted Pella, Inc., Tustin, CA).
2. Fix for 3 h at room temperature swirling occasionally. De-gas (in a dessicator) for 10 min at 1 h and 2 h. When de-gassing, bring the vacuum up and down slowly.
3. Remove the fixative from the vials and wash the tissue with 10 ml of 0.05 M sodium cacodylate trihydrate for 30 min. Swirl occasionally. Repeat.
4. Remove the buffer and add 10 ml of 5% ethanol (in water). Incubate at room temperature for 30 min under constant vacuum. Repeat this for the following ethanol solutions: 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 95%, 100%. Leave the tissue overnight in 100% ethanol, no vacuum.
5. Replace the 100% ethanol with fresh 100% ethanol. Incubate at room temperature for 1 h, no vacuum. Repeat.
6. Remove the 100% ethanol and add 10 ml of 25% xylene (in ethanol). Incubate at room temperature for 30 min, no vacuum. Repeat this for the following solutions: 50% xylene (in ethanol) and 75% xylene (in ethanol), no vacuum. Remove the 75% xylene solution and replace it with 100% xylene. Incubate at room temperature for 1 h, no vacuum. Repeat twice with 100% xylene.
7. Without removing the 100% xylene, add 15 chips of Paraplast Plus (Fisher). Leave it overnight at room temperature.
8. The paraplast will partially go into solution. Incubate the tissue at 42°C to solubilize the remaining paraplast. Add five more chips and incubate at 42°C for 3–4 h. Repeat.
9. Pour off the paraplast/xylene solution. Add 10 ml melted paraplast plus. Incubate at 57–62°C for at least 8 h[b]. Repeat at least six times.

FAA fixation

FAA
50% Ethanol
5% Acetic acid
10% Formalin (3.7% formaldehyde)

1. Carry out step 1 above, except that FAA is used as the fixative instead of glutaraldehyde/cacodylate.
2. Carry out step 2 above.
3. Remove the fixative from the vial and wash the tissue with 10 ml of 50% ethanol (in water) for 30 min under constant vacuum. Repeat.
4. Remove the 50% ethanol solution and add 10 ml of 60% ethanol. Incubate at room temperature for 30 min under constant vacuum. Repeat this for the following ethanol solutions: 70%, 80%, 95%, 100%. Leave overnight in fresh 100% ethanol, no vacuum.
5. Continue with step 5 as above.

[a] The tissue to fluid ratio should never be greater than 1:10. The tissue pieces should be small, 1 mm × 1 mm–1 mm × 10 mm, to facilitate quick fixation.
[b] Do not allow the temperature to exceed 62°C at any time. Temperatures in excess of 62°C will cause breakdown of the polymer structure of the paraffin.

6.3 Pre-hybridization treatments

The pre-hybridization treatments (see *Table 16*) have two aims, to increase the accessibility of the target RNA and to lower non-specific background binding. Efficient hybridization of target RNA after crosslinking fixation requires some protease digestion. Proteinase K and pronase have both been used for this

Table 15. Slide preparation.

1. Incubate the slides in chromerge overnight or longer.
2. Rinse in running distilled water for >1 h.
3. Bake at 200°C to dry.
4. Immerse in 100 μg/ml poly-L-lysine, 10 mM Tris–HCl (pH 8.0) for 10 min at room temperature[a].
5. Loosely cover to keep dust off the slides and allow to air dry.

Sectioning

1. Cut 5–10 μm thick sections and float them on a 45°C waterbath to spread.
2. Mount them on poly-L-lysine-coated slides. Incubate at 40°C overnight.

[a]Gelatin and albumin have been tested as alternative subbing agents for retention of sea urchin embryos. Poly-L-lysine was vastly superior. These alternative subbing agents have not been tested for retention of plant material.

Table 16. Pre-hybridization treatments.

1. Incubate the slides in xylene for 10 min with stirring in a staining dish to remove the paraplast. Repeat.
2. Hydrate the sections by passing the slides sequentially through the following solutions, 100%, 100%, 95%, 85%, 70%, 50% and 30% ethanol and two changes of water. Dip the slides about 20 times in each solution.
3. Incubate the slides in 1% BSA in 10 mM Tris–HCl (pH 8.0) at room temperature for 10 min. This will block the positive charges on the slide from the poly-L-lysine and reduce background.
4. Remove the BSA by washing twice with water in a staining dish.
5. Incubate the slides in 1 μg/ml Proteinase K in 100 mM Tris–HCl (pH 7.5), 50 mM Na_2EDTA in a Coplin jar for 30 min at 37°C. Pre-warm the Tris/EDTA solution before use.
6. Remove the Proteinase K by washing twice with water in staining dishes.
7. Equilibrate the slides in 0.1 M triethanolamine (pH 8.0). Add undiluted acetic anhydride to a dry staining dish, add the slides, then add 0.1 M triethanolamine (pH 8.0) to the dish to give a final acetic anhydride concentration of 0.25% (v/v), mix. Dip the slides several times. Incubate at room temperature for 10 min. This reaction acetylates any remaining positive charges in the tissue or on the slides, further reducing background[a].
8. Wash the slides briefly in a staining dish with 2× SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0).
9. Dehydrate the sections by passing them through 30%, 50%, 70%, 85%, 95%, 100% and 100% ethanol as in step 2[b].
10. Dry under vacuum.

[a]Tissue sections are sometimes lost from the slides at this step. This can be minimized by dipping the slides gently.
[b]All but the 100% ethanol solutions, which are contaminated with xylene, can be re-used.

(23,42). The extent of deproteinization may have to be optimized for individual tissues. The goal in this case is to maximize the hybridization signal without losing morphological structures or causing the sections to fall off the slides during the procedure. We have determined, however, that the published proteinase K digestion procedure for sea urchin embryos (23) also results in maximum hybridization signals when tobacco root tips are analysed. We conclude from this and other studies that the choice of fixative may be more important in setting the proteinase K conditions than the tissue type. The other

pre-treatments, BSA and acetic anhydride, are used to lower non-specific background binding to both the sections and the slides.

6.4 Hybridization

Discussions of probes, hybridization conditions, sensitivity and hybridization kinetics can be found in other sources (23,44,45). The following simply highlights the important features for constructing experiments.

6.4.1 *Choice of probe*

Previous work has demonstrated that single-stranded probes result in higher signals than double-stranded probes (23). We favour single-stranded RNA probes over DNA probes for the following reasons.

(i) Post-hybridization RNase digestion allows removal of unbound probe without loss of signal.
(ii) The higher thermal stability of RNA–RNA duplexes permits higher wash temperatures, resulting in lower non-specific probe background.

The simplest way to generate single-stranded RNA probes is using the SP6-T7 *in vitro* transcription system which has been discussed previously (Section 4).

Probes can be labelled with ^{3}H, ^{32}P or ^{35}S. ^{3}H results in the highest resolution as well as the lowest background levels at equal specific activity. However, ^{3}H has a very low autoradiographic efficiency (46) and requires extremely long incubation periods to get high grain density for all but the most abundant RNAs. ^{32}P-labelled probes can be very high in specific activity; however, the resolution is usually insufficient to localize expression on a cellular level (45). We favour ^{35}S-labelled probes (see *Table 17*). Using ^{35}S, one can obtain specific activities 10-fold higher than ^{3}H, and the autoradiographic efficiency is about five times higher. Resolution using these probes is sufficient to assay RNA accumulation in individual cells. The hybridization procedure shown in *Table 18* has been modified for use with ^{35}S-labelled probes.

After synthesis the probe is partially hydrolysed to 100–200 nt. Several laboratories have shown that short probes result in higher signals (23,47).

6.4.2 *Probe concentration*

Choosing the correct probe concentration is important to maximize sensitivity. It has been shown previously that non-specific probe binding increases approximately linearly as a function of probe concentration (23). Therefore, maximum signal/noise ratios will be obtained at a probe concentration close to target sequence saturation. Saturation concentrations have been determined for several probes hybridized to sea urchin embryos (48). As predicted, the probe concentration required for saturation is proportional to its sequence complexity. About 0.3 μg/ml probe per kb of probe complexity is required. Similar saturation analyses have not been made in any plant systems.

Table 17. Probe preparation.

We synthesize ^{35}S-labelled RNA probes with specific activities ranging over 1–5 × 10^8 d.p.m./μg. Procedures for synthesizing these probes can be found in Section 4, *Table 10* (steps 1–6) and *Table 11* (steps 1–5,7).

Alkaline hydrolysis of probes

1. Resuspend the probe in 50 μl of H_2O.
2. Add 30 μl of 0.2 M Na_2CO_3 and 20 μl of 0.2 M $NaHCO_3$.
3. Incubate at 60°C for the calculated time.
   $t = (L_o - L_f)/(KL_oL_f)$
   L_o = starting length (kb)
   L_f = final length (kb) (we suggest 0.15 kb)
   $K = 0.11$
   Calculated time is in minutes.
4. Stop the reaction by adding 3 μl of 3 M sodium acetate (pH 6.0) and 5 μl of 10% glacial acetic acid.
5. Add 1/10 vol. of 3 M sodium acetate and 2.5 vols of ethanol. Ethanol precipitate at −20°C overnight.
6. Size the probes on a glyoxyl gel (49).

6.4.3 *Hybridization criteria*

The optimum temperature for *in situ* hybridization using homologous RNA probes of average GC content (50%) in the buffer listed in *Table 18* is 45–50°C (23). This is 25°C below the T_m for duplexes formed *in situ*.

Hybridizations have been done using heterologous probes (23). The decrease in thermal stability for hybrids formed *in situ* was shown to be the same as for hybrids formed in solution, that is 1% sequence divergence lowers the T_m by 1°C.

6.5 **Post-hybridization treatments**

6.5.1 *Post-hybridization washes*

We use a combination of RNase digestion and low salt–high temperature washes to lower non-specific background binding (*Table 19*). RNase digestion has been shown to be essential for identifying hybridization patterns over background. If the signal/noise ratio is low, it may be possible to raise the stringency of the final wash.

The original wash procedure shown in *Table 19* has been modified (Robert Angerer, personal communication). That wash procedure is also shown in *Table 19*. The wash temperature is only about 10°C below the *in situ* T_m for a probe of average GC content. One should be cautious when using this procedure with AT-rich probes.

6.5.2 *Autoradiography*

We carry out all steps involving the emulsion in absolute darkness (*Table 20*). However, it has been reported that the 'Duplex Super Safelight' consisting of

Table 18. Hybridization.

Final concentrations in the hybridization mix are: 50% formamide, 300 mM NaCl, 10 mM Tris–HCl (pH 7.5), 1 mM Na_2EDTA, 1× Denhardt's (0.02% BSA, 0.02% Ficoll and 0.02% PVP), 10% Dextran sulphate, 100 mM DTT, 25 U/ml RNasin, 500 μg/ml poly(A) and 150 μg/ml tRNA.

1. Combine the probe[a], tRNA, poly(A) and one half of the DTT in water. Incubate at 80°C for 5 min. Add the rest of the hybridization components.
2. Add 100 μl of hybridization mix to each slide.
3. Cover the sections with a 22 × 60 mm silanized, baked (>200°C, 2 h) coverslip[b].
4. Immerse the slides in pre-warmed mineral oil and incubate at the appropriate temperature for 16 h.

[a]The final probe concentration should be 0.2–0.5 μg/ml per kb of probe complexity.
[b]Saturate the sections with the hybridization mix before applying the coverslip or bubbles will form in the tissue. The easiest method to apply the coverslip is to hold it at an angle with a pair of electron microscope forceps and slowly lower the coverslip onto the slide. Avoid bubbles.

Table 19. Post-hybridization treatments.

Method 1

1. Drain the excess oil from the slides. Pass the slides through three changes of chloroform in Coplin jars taking care that the coverslips remain on and that the slides are free from oil. Let the chloroform evaporate from the slides.
2. Incubate the slides in 4× SSC/5 mM DTT at room temperature for about 5 min or until the coverslips fall off (1× SSC is 150 mM NaCl, 15 mM sodium citrate). Pass the slides through two more changes of 4× SSC/5 mM DTT. This removes the bulk excess probe. Store in fresh 4× SSC/5 mM DTT until all of the slides have been processed.
3. Incubate the slides in 50 μg/ml RNase A in 0.5 M NaCl, 10 mM Tris–HCl (pH 7.5), 1 mM Na_2EDTA for 30 min at 37°C. Pre-warm the buffer to 37°C before beginning the incubation.
4. Wash in RNase buffer/5 mM DTT at 37°C for 20 min. Repeat three more times.
5. Place the slides vertically in a test tube rack and wash with stirring in 5 litres of 2× SSC/1 mM DTT for 30 min at room temperature.
6. Wash the slides with stirring in 5 litres of 0.1× SSC/1 mM DTT for 1 h at the appropriate temperature. A wash temperature of 45–50°C is roughly equivalent to hybridization criteria of 45°C, 50% formamide, 300 mM Na^+[a].
7. Dehydrate the tissue by passing it through the following filtered solutions (0.45 μm Millipore filter) in staining dishes: 30%, 50%, 70%, 85%, 95% ethanol each containing 100 mM ammonium acetate and 100%, 100% ethanol. Dip the slides 20 times each. Dry under vacuum at room temperature.

Method 2

1. Clean the slides as described in step 1 and 2 above.
2. Dehydrate the tissue by passing it through graded ethanols containing 300 mM ammonium acetate: 30%, 50%, 70%, 85% and 95%. Pass the slides through two changes of 100% ethanol.
3. Incubate the slides in hybridization buffer [50% formamide, 0.3 M NaCl, 20 mM Tris–HCl (pH 7.0), 1 mM Na_2EDTA (pH 8.0), 10 mM DTT] at 55–65°C for 10 min.
4. Transfer the slides to 0°C 2× SSC/10 mM DTT and continue as described above from step 3.

[a]We routinely wash the slides at 57°C. This high stringency wash reduces non-specific background without significantly lowering the signal.

Table 20. Autoradiography.

The slides are dipped in NTB-2 Liquid Track Emulsion (Kodak) that has been diluted 1:1 with 600 mM ammonium acetate and aliquoted. The aliquots contain 10 ml of emulsion and are stored in light-tight containers at 4°C. We carry out all darkroom work in absolute darkness.

1. Melt an aliquot of emulsion in a suitable dipping container in a 45°C waterbath. This takes about an hour.
2. After the emulsion has warmed to 45°C, dip the slides by immersing once as smoothly as possible.
3. Holding the slides vertically, blot the bottom edge on a paper towel. Place the slides vertically in a test tube rack to dry. Allow the slides to dry for 1 h at room temperature.
4. Transfer the slides to a black slide box containing a tube of desiccant. The slides are exposed at 4°C in a light-tight box.

Developing

1. Develop the slides for 2.5 min in D-19 developer (Kodak) at 15°C. Dip the slides up and down five times then let the slides sit for a total of 2.5 min*.
2. Stop in 2% acetic acid at 15°C for 30 sec dipping continuously.
3. Fix in Kodak Fixer (not Rapid Fix) at 15°C. Dip up and down five times. Let the slides sit in fixer a total of 5 min.
4. Rinse in distilled water.
5. Rinse in cold running water for 15 min.

Staining

1. Stain the slides in 0.1% toluidine blue (water) for ~1 min. Do test slides for each tissue because the amount of time necessary for optimal staining varies.
2. Rinse quickly with water.
3. Dehydrate the tissue by passing the slides quickly through the following solutions: 25%, 50%, 75%, 100% and 100% ethanol.
4. Dip in xylene.
5. Add a few drops of permount. Wipe the coverslip while pressing down to remove excess xylene/permount. Let the slides dry overnight.

*Longer developing times and higher temperatures preferentially produce grains in the emulsion background.

FDY filters in the top slots and FDW filters in the bottom slots did not increase background emulsion grains after a 3 h exposure (45).

6.5.3 *Exposure time*

We have analysed RNA sequences present at 10–0.1% of the poly(A^+) RNA. Using ^{35}S-labelled probes of the specific activity discussed previously, high grain densities (e.g. see *Figure 1*) were obtained after 1–21 days of exposure. Of course exposure time must be determined empirically for each new probe. Grain density depends on the target RNA concentration per cell which cannot be predicted from alternative molecular analyses.

6.5.4 *Staining*

There are many staining procedures for plant tissue. We have used toluidine blue because it provides good differentiation between cytoplasm and nucleus,

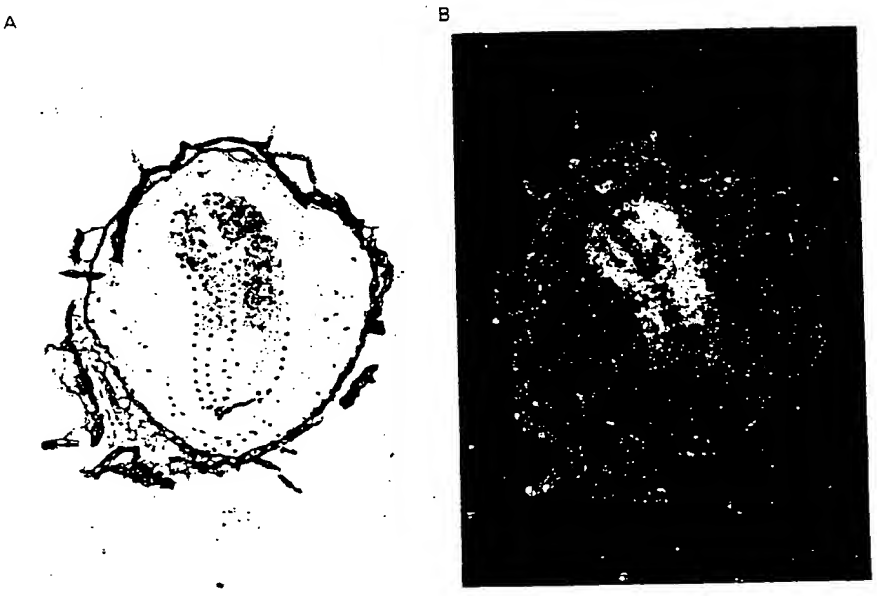

Figure 1. Localization of β-conglycinin mRNA in transformed tobacco seeds. Seeds, 19 days after pollination, were fixed in FAA, embedded in paraffin, and cut into 10 μm sections. A ^{35}S-labelled anti-sense RNA probe (1.5×10^8 d.p.m./μg) was hybridized to plant sections for 14 h at hybridization criteria of 42°C, 0.3 M Na^+ and 50% formamide. Wash criteria were 57°C and 0.02 M Na^+. The slides were developed after 3.5 days of exposure. Seed sections were stained with 0.5% toluidine blue. (A) Longitudinal section of a tobacco seed. The photograph was taken using bright-field microscopy. (B) Hybridization with a ^{35}S-labelled anti-sense probe. The photograph was taken using darkfield microscopy. The hybridization signal can be seen over embryo cotyledon cells and upper axis cells. [Reproduced from Barker *et al.* (37).]

and yet stains uniformly enough not to interfere with visualization of silver grains under dark field illumination.

6.6 **Quantitative analysis**

It has been shown that 3- to 8-fold differences in RNA concentrations in sea urchin embryos were accurately reflected in relative grain densities using this *in situ* hybridization procedure (23). We use these results to suggest that data derived from this technique may be interpreted quantitatively as well as qualitatively with the following caveats. In the two experiments described above, comparisons were made using a single probe hybridized to RNA in the same or very similar cell types. It remains possible that different cell types may have different RNA retention or hybridization efficiency. Second, in order to compare signals with the greatest degree of accuracy, the sections being examined should be from the same experiment and on the same slide. We have observed slight quantitative differences in signals from experiment to experiment and from slide to slide.

7. ACKNOWLEDGEMENTS

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Ilan Sela and Sylvia Zeitoune-Simovich

Serial No.: 09/889,821 Int'l Appln. No.: PCT/IL00/00029

Filed : July 18, 2001 Int' Filing Date: January 16, 2000

Art Unit : 1638 Examiner: A. Mehta

For : AN EXPRESSION SILENCING SYSTEM AND DIFFERENT USES THEREOF

1185 Avenue of the Americas
New York, New York 10036

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION UNDER 37 C.F.R. §1.132

I, Ilan Sela, hereby declare that:

1. I am one of the inventors of the invention claimed in the above-identified patent application.

2. I am an expert in the field of expression-silencing systems and uses thereof. Attached hereto as **Exhibit 1** is my *curriculum vitae*.

3. I have reviewed the subject patent application, including the pending claims and the March 18, 2004 Office Action.

4. I understand that in the March 18, 2004 Office Action, the Examiner rejected claims 1-7, 9, 11, 13-25 and 27-30 as allegedly not enabled. Specifically, the Examiner

contends that the specification does not enable the claimed invention when the target sequence is a non-coding sequence.

5. Example 5 of the subject application shows how the use of the TMV translation enhancer Ω element, a non-coding sequence, can be efficiently used as the targeting sequence in the silencing system described by the invention. Cloning of the 68 nucleotide TMV Ω element sequence downstream of the T7 promoter and its transformation to a plant expressing the T7 RNA polymerase, conferred resistance to TMV infection. As shown in Table 2 of the subject application, the virus titer (as measured by ELISA) was significantly reduced in the plants double transformed with the silencing system.

6. These results stem from the inability of the virus to replicate as shown in Figure 1 of Appendix A (attached hereto as **Exhibit 2**). Dot blot analysis of TMV-infected protoplasts generated from non-transgenic plants and from silenced plants (using a TMV probe sequence) clearly shows increasing viral amounts in the non-transformed samples 48 hours post-infection. In the double transformed protoplasts, the signal remained at background level even after 72 hours post-infection, proving that the virus did not replicate and that the silencing effect at the cellular level is instantaneous.

7. Furthermore, TMV resistance induced by the silencing system of the claimed invention prevails over time (11 weeks) as shown in Figure 2 of Appendix A (attached hereto as **Exhibit 2**).

8. At the plant level, following a brief stage of cellular silencing, the virus is able to overcome silencing by exerting its silencing suppressor capacity and replicate normally for a certain period (2-7 weeks). Later on, the plant's silencing mechanism overcomes the virus' effect and the viral infection declines. This plant phenomenon is acknowledged as "viral infection recovery."

9. Therefore, the data provided in the specification as filed and in Appendix A show that silencing can be induced by the system of the invention, using a non-coding nucleotide sequence as the targeting sequence.

10. The silencing induction with the subject system is very efficient at the cellular level and it persists over time.

11. Further experiments performed also show the claimed silencing system as versatile and very efficient in addition to being enabled. According to the data provided in Appendix B (attached hereto as **Exhibit 3**), T7-derived RNA-silencing of endogenous genes in plants can be achieved with 80-100% efficiency, when using even gene fragments (300-400 bp).

12. Silencing can be attained by transforming one group of plant cells with a first construct harboring the gene for T7-pol, transforming another group of plant cells with a second plasmid carrying even part of a target gene placed between pT7 and the T7 and *nos* terminators, crossing transformed plants from the first group with transformed plants from the second group and selecting for double transformed progeny (as demonstrated in the Tomato (*Lycopersicon esculentum*) example in Appendix B attached hereto as **Exhibit 3**).

13. Silencing can also be attained by transforming plant cells with the plasmid carrying the gene for T7 RNA polymerase (T7-pol) placed between the 35S constitutive promoter and the *nos* terminator. Plants expressing the *T7-pol* are re-transformed with another plasmid carrying a nucleotide targeting sequence placed between the T7 promoter (pT7) and the T7 and the *nos* terminators, and double-transformed plants are selected (as demonstrated in the Tobacco *Nicotiana N* gene silencing example in Appendix B attached hereto as **Exhibit 3**).

14. Using both approaches, gene silencing was efficiently accomplished.

15. When *V. dahliae*-resistant, non-transformed VF-36 plants and doubly-transformed VF-36 tomato plants were challenge-inoculated with a virulent isolate of *V. dahliae* race 1, the doubly transformed VF-36 plants

lost their resistance. The transformed plants became *Verticillium*-susceptible and remained stunted, most of them eventually dying. Tomato plants, silenced for *Ve*1, lost their resistance to *Verticillium*. These phenotypic changes were noted in 100% of the *Ve*-silenced tomato plants.

16. Tobacco plants carrying the *N* gene (tobacco^{NN}) react to TMV infection by producing local necrotic lesions restricted to sites around the point of virus entry. When leaves of the doubly transformed plants, silenced for the N gene were inoculated with TMV, necrosis spread beyond the local lesions in about 80% of the transformed plants. TMV did not spread and remained confined to the site of infection. The T7-derived silencing system lead to a phenotypic change with respect to the spread of necrosis.

17. T7-derived RNA-silencing of endogenous *Ve*1 and *N* genes was not only detected at the molecular level, but was also manifested by phenotypic changes.

18. The T7-derived silencing system is not limited to transgenes such as *GUS* or exogenous pathogens such as TMV. The T7-derived silencing system also successfully silenced endogenous genes like the tobacco *N* gene and the tomato *Ve*1 gene.

19. I also understand that in the March 18, 2004 Office Action, the Examiner rejected claims 1-7, 9, 13-17, 19-

25 and 27-30 as allegedly obvious over Lassner et al. in combination with Blockland et al. (Plant J., 1994, vol.6, pages 861-877) and Palauqui et al. (EMBO J., 1997, vol. 16, pages 4738-4745).

20. The claimed T7-derived silencing system holds features different from other silencing systems described in the cited literature, as detailed in Appendix C (attached hereto as **Exhibit 4**).

21. T7-derived silencing is a type of RNA silencing. However, several parameters associated with the silencing pathway in plants were not observed. No siRNA could be detected in total RNA extracts of silenced plants. The silencing signal was not transduced across grafts. The viral silencing suppressor HC-pro could not overcome the T7-derived silencing effect. Yet, the silenced genes were methylated at their coding regions, pertinent siRNAs were detected only in nuclear extracts and dicer activity was enhanced in silenced plants. The T7-derived silencing system activity seems to be confined to the nucleus.

22. I understand that the Examiner asserts that Blokland et al. teach co-suppression of pigmentation of chalcone synthase (chs) in Petunia plants comprising a construct comprising the CaMV 35S promoter operably linked to a nucleotide sequence encoding a fusion of the uidA (GUS) gene and the chsA cDNA. Steady state chs mRNA levels, and pigmentation, were reduced in the transgenic

plants, including in flowers. GUS expression was also silenced in plant parts that were also silenced for chs. The construct used also comprised nos terminator operably linked to the uidA/chs coding sequence (pages 862-866).

23. Certain aspects of the silencing process described by Blockland et al. are distinct from the process of the claimed invention. One of the most important is the difference in the efficiency of the system. While Blockland et al. show about 8% efficiency (1 out of 12-15), the T7-driven silencing system is 80-100% efficient (see Appendix B attached hereto as **Exhibit 3**).

24. Blockland et al. demonstrate that silencing of the *chs* genes in petunia is not always associated with highly transcribed *chs* transgenes. This suggests that the absolute level of normal mRNA is not important. As shown in Appendix B (attached hereto as **Exhibit 3**), TMV infection considerably stimulates *N* expression in tobacco^{NN} and the degree of silencing was proportional to the level of the expressed *N mRNA*. Leaves with the highest levels of *N mRNA* exhibited the strongest silencing. The degree of RNA silencing is dependent of the level of the mRNA.

25. Blockland et al. state that the "suppressed *chs* genes and transgenes are transcribed seemingly unaltered; indicating that silencing does not result from a type

of DNA modification that prevents transcription initiation." They also were not able to detect differences between the methylation status of suppressed and non-suppressed genes. As described in Appendix C (attached hereto as **Exhibit 4**), a difference in the *Hpa*II cleavage pattern was observed between silenced and non-silenced plants, indicating that a CCGG sequence at the NBS domain of *N* was methylated. Methylation density analysis and cytosine methylation test (using the bisulfite method) *in GUS*-silenced plants show a dense DNA methylation at the *GUS* coding region. In the silenced plants, cytosine methylation was not restricted to GC or GNC sites.

26. Another unique characteristic of the T7-driven silencing system is the capacity of using only a short homology fragment (around 70 nucleotides) as the targeting sequence in order to successfully induce and preserve gene expression silencing as shown in the TMV translation enhancer Ω element experiments in Appendix A (see **Exhibit 2** attached hereto).

27. The Examiner states: "Palauqui et al. teach that silencing is transmitted with 100% efficiency from silenced rootstock, derived from transgenic plants that were co-suppressed for nitrate reductase and the uidA transgene, to grafted scions that were from transgenic plants that nitrate reductase and uidA (pages 4739-4742)."

28. In plants, usually long-distance silencing signals engender silencing in non-silenced scions upon grafting on silenced rootstocks as described in Palauqui et al.

29. In the T7-derived silencing system a lack of long-distance signaling was observed (see Appendix C, Table 1, attached hereto as **Exhibit 4**). Doubly-transformed plants, silenced for *GUS*, were grafted with scions harboring and expressing 35S-*GUS* without silencing *GUS* expression in the scions. Reciprocally, GUS expression also occurred when the 35S-*GUS* plants served as rootstocks on which the *GUS*-silenced doubly transformed plants were grafted. To rule out the possibility that lack of signaling between rootstock and scion was due to inept grafting technique, TMV-infected scions were grafted on healthy plants, and demonstrated the virus' spread across the graft. Also demonstrated was that in the case of intron-spliced hairpin silencing of *GFP*, a long-distance signal is transduced across the graft, engendering silencing in GFP-expressing scions (data not shown).

30. In the T7-driven silencing system, silencing is restricted to the cells carrying the constructs. There is no transmission of the signal to other tissues as seen in *C. elegance* or in plants, as described by Palauqui et al.

31. Finally, the Examiner asserts that "it would have been obvious and within the scope of one ordinary skill in

the art at the time the invention was made to use the RNA polymerase/pT7 system of Lassner et al. to silence gene of interest in plant cells, for example the chs gene of Blockland et al. It was obvious that the constructs comprising the T7 RNA polymerase coding sequence, and pT7-target sequence could have been introduced into the same plant cell, and a transgenic plant regenerated therefrom; or into separate plant cell, wherein two transgenic plants would have been regenerated therefrom, and subsequently crossing the two plants to bring the two constructs into the same plant. Whether both DNA constructs were introduced into the same plant initially, or into different ones which were subsequently crossed, amounts to an optimization of process parameters. It further would have been obvious that crossing the plant containing both DNA constructs to a non-transgenic plant that expresses the target sequence, would result in progeny plants that comprise both DNA constructs, and that the target sequence would have become silenced. It further would have been obvious to graft a plant, in which the target sequence, for example chs, expressed its product, onto a rootstock from the transgenic plant comprising the two DNA constructs and wherein the targeting sequence, for example, was from the chs coding sequence. It would have been obvious, given the teaching of Palauqui et al., that the chs coding sequence would have become silenced in the grafted scion. One would have been motivated to use the T7 RNA polymerase/pT7 system to express a target in sequence to silence a target

sequence, given the teaching of Lassner et al. that this system successfully allows the transcription of nucleotide sequences operably linked to pT7 when in plant cells."

32. The T7-driven silencing system of the application comprises two constructs: T7 RNA polymerase and T7 promoter-target sequence. These two constructs acting together engender silencing of transgenes, endogenous and exogenous pathogenic genes. The silencing induction is very efficient (≅100%) and a small targeting sequence (as small as 70bp) is sufficient to induce and preserve gene expression silencing. This system's silencing process is a post-transcriptional event but also involves DNA methylation. Its activity is constrained to the transformed cells and it cannot be transmitted from stocks to grafted scions.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made herein on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the subject application or any patent issuing thereon.

Dated: _____ _____

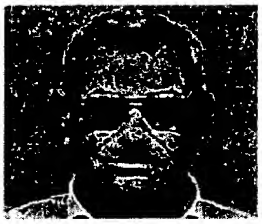
Ilan Sela

I hereby declare that all statements made herein of my own knowledge are true and that all statements made herein on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the subject application or any patent issuing thereon.

Dated: Sept 12, 04

Ilan Sela

The Robert H. Smith Institute of Plant Sciences and Genetics in Agı



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Teaching
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Kennedy-Leigh Center

Tel: 972-8-948-9098
Fax: 972-8-948-9899
E-mail:
bergere@agri.huji.ac.il

Ilan Sela

Professor of Virology and Molecular Biology

Born: 1936
Ph.D. (1964): Antiviral mechanisms in virus-infected plants.
Sabbaticals and training outside of Israel: The University of Wisconsin, Madison, De| Biophysics (1965-67); The Medical College of Wisconsin, Milwaukee, Dept. of Bioch (1973-4); Roche Institute of Molecular Biology, New Jersey (1983-4); Walter Reed A Washington D.C., Dept. of Cellular Immunology (1990-91); Crown research Institute, Science, Auckland, New Zealand (1995; 2000). The University of California, Davis, D Sciences (2000).

Full professor since 1984.

The Hebrew University of Jerusalem
Faculty of Agricultural, Food Quality and Environmental Sciences
Robert H. Smith Institute of Plant Sciences and Genetics
Virus Laboratory
Rehovot 76100
Israel

Phone: +972-8-9489377
Fax: +972-8-9473402
e-mail: sela@agri.huji.ac.il

Updated: December 2003.

Main Research Interests

- RNA silencing of plant and viral gene expression.
- Transgenic plants
- Grapevine viruses.
- Viruses of bees.
- The phytoplasma genome.

Current research activities:

- RNA silencing of endogenous genes in plants by a unique T7-driven system.
- Silencing of viral genes.

Applicants: Ilan Sela et al.
Serial No.: 09/889,821
Filed: July 18, 2001
Exhibit 1

- Plant genes involved in RNA silencing.
- Nuclear activities of the T7-derived silencing mechanism.
- The molecular genetics of a temperature related necrosis phenomenon in to
- The deciphering of a bee-virus genome.
- The deciphering of the phytoplasma genome.
- Phytoplasmal surface proteins.
- Grapevine viruses.

Teaching:

Introduction to Molecular Biology (71065) – An undergraduate course.
Experimental Aspects in Molecular Biology (71957) – A graduate course.
Laboratory Training in Molecular Biology (71177) – A graduate course.
Virology (71953) – a graduate course.
Biotechnology for Law Students (62224). – All levels.

Graduate Students:

Currently supervising 6 "Master" students and 5 Ph.D. students.
Total students supervised along the years: 60 "Master" students and 20 Ph.D. stude
at the Post Doctoral level: 11 people.

Current research grants:

RNA silencing in plants. Part of a Magnet program within the TEVEL consortium.
Bee Viruses. A BARD-supported project.

Previous research grants were provided by:

University internal grants, Volkswagen Foundation, BSF, NIH, Roche, BARD, GIF, Ir
sources in Israel.

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(Out of 95 review papers)

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Appendix A

TMV infection plant recovery: usage of the Ω translation enhancement element as the target sequence in the silencing system.

Figures legend

Figure 1. TMV Dot blot hybridization.

RNA samples from non-transgenic protoplasts and from silenced protoplasts inoculated with TMV were collected along time (0, 24, 48 and 72 hours post-inoculation) and hybridized with a TMV probe.
Upper row: RNA samples extracted from non-transgenic protoplasts infected with TMV. Lower row: RNA samples extracted from double transformed silenced protoplasts infected with TMV.

Figure 2. TMV duplication in Tobacco infected leaves.

TMV titer as measured by ELISA using anti-TMV antibodies in random selected plant leaf discs during a period of 11 week post-inoculation (1, 2, 7 and 11 weeks).
DT: double transformed (with Ω TMV) silenced plants; SR1: control plant; pT7GUS: double transformed plant silenced for GUS; 35ST7pol: single transformed plant expressing the T7 RNA polymerase.

TMV Dot Blot Hybridization

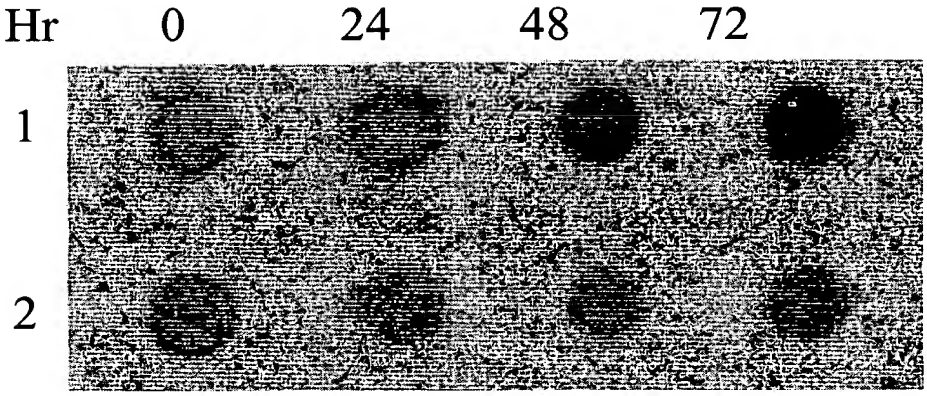

Figure 1

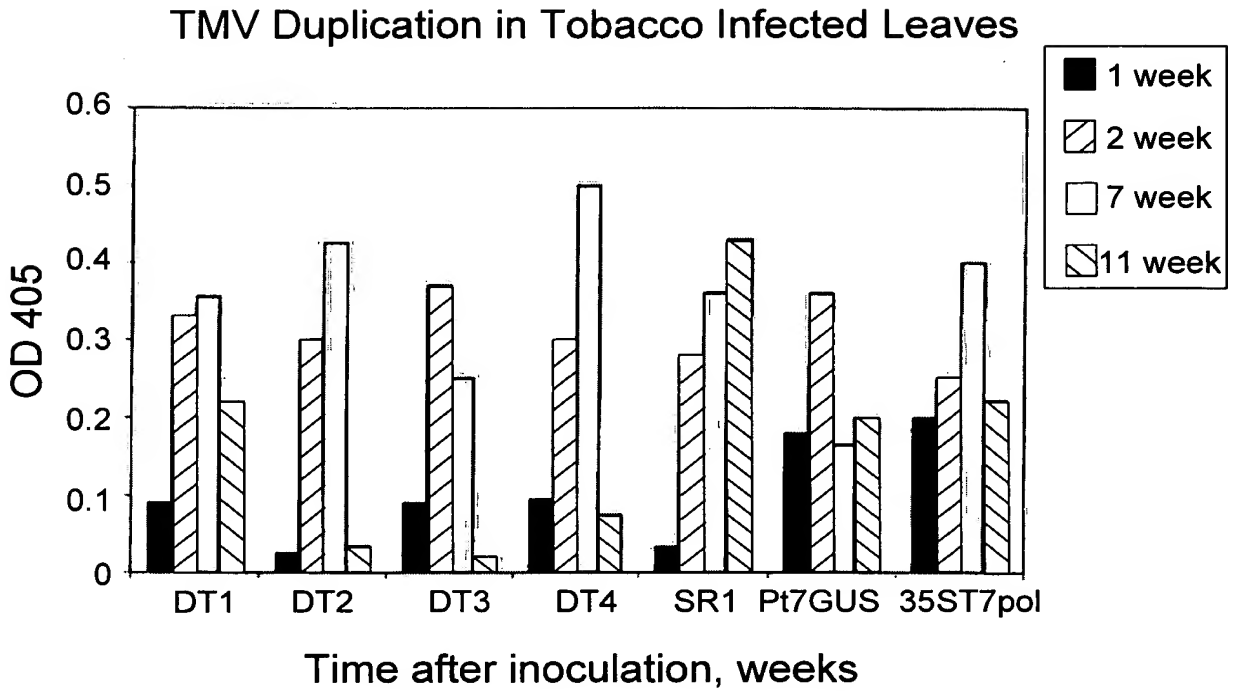
TMV Duplication in Tobacco Infected Leaves
1 week
2 week
7 week
11 week
OD 405
0.6
0.5
0.4
0.3
0.2
0.1
0
DT1
DT2
DT3
DT4
SR1
Pt7GUS
35ST7pol
Time after inoculation, weeks

Figure 2

Appendix B

Silencing of the Tobacco *N* Gene and the Tomato *VE* Gene by a T7-Driven RNA-Silencing System

Michal Levy, Eva Avisar, Orit Edelbaum, Haim Rabinowitch, and Ilan Sela[1]

The Hebrew University of Jerusalem, Robert H. Smith Institute for Plant Science and Genetics, Faculty of Agriculture, Rehovot 76100, Israel.

[1]Corresponding author. E-mail sela@agri.huji.ac.il; telephone: +972-8-9489377; fax: +972-9743402.

Running title: T7-driven silencing of endogens.

The corresponding author is responsible for distribution of materials integral to the findings presented in this article.

Michal Levy and Eva Avisar contributed equally to this paper.

Silencing of the Tobacco *N* Gene and the Tomato *VE* Gene by a T7-Driven RNA-Silencing System

ABSTRACT

Previously, we described a T7-derived RNA-silencing system in plants. In that system, expression of the transgene of the reporter gene *GUS* was silenced when placed downstream of the T7 promoter in transgenic plants already expressing T7 RNA polymerase (Zeitoune et al., 1999). The accompanying paper (Meir et al.) demonstrates that this type of silencing is unique and does not entirely conform to the present consensus paradigm of RNA silencing. The present paper describes the T7-driven RNA silencing of two endogenous resistance genes: the *N* gene, conferring TMV resistance in tobacco, and the tomato *Ve*1 gene, conferring resistance to the fungus *Verticillium dahliae*. In both cases, we introduced a ca. 300- to 400-bp fragment of the pertinent gene into a plant expressing T7 RNA polymerase, downstream of the T7 promoter. These transformations resulted in distinct phenotypic changes. *N*-gene-silenced tobacco ceased to restrict necrosis to local lesions; however, it still restricted the spread of TMV. Tomato plants, silenced for *Ve*1, lost their resistance to *Verticillium*. These phenotypic changes were noted in over 80% of the *N*-silenced tobacco plants and in 100% of the *Ve*-silenced tomato plants. Quantitative RT-PCR analyses indicated a considerable decrease in the level of the respective RNAs in the silenced plants. The reduction in RNA levels brought about by silencing was sufficient to cause pronounced phenotypic modifications, but not totally abolish RNA expression.

INTRODUCTION

Previously, we showed that *in planta* transcription by T7 RNA polymerase (T7-pol) from a T7 promoter (pT7) of the reporter gene *GUS* leads to silencing (Zeitoune et al., 1999; Meir et al., accompanying paper). Since *GUS*-RNA was transcribed and its expression was silenced at a later stage, the T7-driven silencing was regarded as a type of post-transcriptional gene silencing (RNA silencing).

The accompanying paper (Meir et al.) demonstrates that the mechanism leading to T7-driven RNA silencing differs in several aspects from the current consensus model for RNA silencing: no detectable siRNA could be found in the cytosolic fraction, there was no transduction of signals for silencing across a graft, and the silencing was not suppressed by the viral suppressor HC-pro. On the other hand, siRNA was found in the nuclei of silenced plants, DNA methylation of the silenced gene was observed, and stimulation of dicer activity was noted. We hypothesized that this type of silencing mechanism is confined to the nucleus, or the nucleus serves as a sink for siRNA.

The T7-derived silencing of *GUS* could have resulted from aberrant transcription of a foreign gene under artificial circumstances. The lack of common cytosolic denominators with known RNA-silencing elements may imply that the abnormal *GUS* transcript is simply devoid of processing and/or transport signals, and thus remains confined to the nucleus. If this is the case, non-expression of *GUS* cannot be attributed to an active RNA-silencing process. The occurrence of silencing elements (sequence-specific siRNA, DNA methylation of the silenced gene) in nuclei of "silenced" plants (Meir et al., accompanying paper) is only indicative of a silencing process. To determine whether the artificial T7 system engenders a new, more general RNA-silencing pathway in plants, we attempted to silence endogenous genes which, under normal circumstances, produce correct mRNAs and proteins.

In plants, resistance genes (*R*-genes) are constitutively expressed. The *R*-genes' products interact with the products of the pathogens' avirulence genes, initiating cascade pathways that lead to resistance (the gene-for-gene model; Flor, 1942, 1956; Keen, 1992). Since these are clear-cut cases of a single dominant gene determining a phenotype, *R*-genes were selected for our silencing attempts.

Resistance to *Verticillium* wilt has been incorporated into most commercial tomato varieties and has proven to be very durable (Diwan et al., 1999). There are two closely linked inverted *Ve* genes that independently confer resistance to the same pathogen (Kawchuk et al., 2001). The *Nicotiana N* gene, introgressed into tobacco from *N. glutinosa*, confers resistance to tobacco mosaic virus (TMV; Holmes, 1938). Tobacco plants carrying the *N* gene (tobacco^{NN}) react to TMV infection by producing local necrotic lesions. Tobacco is a tetraploid plant, and the genetic constitution of tobacco^{NN} is *NNnn* (Sela, 1981). The *N* gene was isolated and characterized and was found to belong to the TIR-NBS-LRR *R*-gene family (Whitham et al., 1994). In a previous paper, we demonstrated the considerable stimulation of *N*-gene expression in tobacco^{NN} following TMV infection, in both locally infected leaves and the upper, non-infected leaves (Levy et al., in press).

This paper demonstrates that both genes can be silenced at the RNA level by the T7-driven silencing system, and that following silencing, relevant phenotypic changes occur.

RESULTS

Phenotypic evidence of *Ve* silencing

The tomato cultivar VF-36, resistant to *Verticillium dahliae* infection, was transformed with plasmid pGA643-35S-T7 RNA (Zeitoune et al., 1999). These plants expressed T7-pol, and were kanamycin-resistant. Another group of VF-36 tomato plants were similarly transformed with a fragment of the *Ve1* gene (pT7*Ve*), as described in Methods. The *Ve1* fragment was placed downstream of pT7 and introduced into a binary vector carrying a gene for hygromycin resistance. Following *Agrobacterium*-mediated transformation, the resultant kanamycin-resistant and hygromycin-resistant transgenic plants were crossed, and those resistant to both kanamycin and hygromycin were selected. The presence of the *T7-pol* and pT7*Ve* sequences in the selected plants was confirmed by PCR.

V. dahliae-resistant, non-transformed VF-36 plants, as well as the aforementioned doubly-transformed VF-36 tomato plants, were challenge-inoculated with a virulent isolate of *V. dahliae* race 1 (see Methods). As can be seen in Figure 1, non-transformed VF-36 plants were resistant to infection while the doubly

transformed VF-36 plants lost their resistance. The transformed plants became *Verticillium*-susceptible and remained stunted, most of them eventually dying. Brown fungal hyphae were observed in the xylem upon cross-sectioning shoots of the transformed plants. These hyphae were isolated, grown in culture and identified as *V. dahliae* (see Methods), proving that the cause of stunting and death in the transformed plants was indeed infection with that pathogen. In some cases, pT7*Ve*-transformed plants were *Verticillium*-diseased and remained stunted, but did not die.

Molecular verification of *Ve*-RNA silencing

Ve-RNA silencing was tested by quantitative RT-PCR assays for the levels of *Ve*-RNA in non-transformed and transformed VF-36 plants. Quantitative RT-PCR assays were conducted as described in Methods. Samples were removed at the end of various PCR cycles and analyzed by gel electrophoresis. The same RNA preparations were similarly tested for actin, serving as an internal housekeeping control for gene expression. The level of RNA in both PCR assays (with RNA from non-transformed and transformed VF-36 plants) was similar, since the RT-PCR assay for actin gave rise to a visible band at cycle 30 with RNA extracts from non-transformed as well as pT7*Ve*-transformed plants. However, the two sets of plants differed in their level of *Ve*-RNA: the pertinent band appeared in the non-transformed VF-36 plants at cycle 24 and in the silenced, pT7*Ve*-transformed plants only at cycle 36 (Fig. 2).

Phenotypic evidence for *N*-gene silencing

Tobacco^{NN} plants were transformed with a binary plasmid carrying the gene encoding T7-pol between the CaMV-35S promoter and the *nos*-terminator, as well as the *NPTII* gene as a kanamycin-selectable marker. These plants expressed T7-pol, as determined by RT-PCR and western blot analyses (data not shown). Plants carrying *T7-pol* were then further transformed with a second construct carrying a segment of the nucleotide-binding site (NBS) region of the *N* gene (pT7*N*) as described in Methods. The second construct carried a gene for hygromycin resistance. Plants resistant to both kanamycin and hygromycin were selected, and the presence of *T7-pol* along with the cloned segment of NBS was verified by PCR. The various constructs and the methods of transformation are described in Methods.

One leaf of every doubly transformed plant was inoculated with TMV. In about 80% of the infected doubly transformed plants, necrosis spread beyond the local

lesions. Various stages of necrosis were observed: first, in the inoculated leaf, necrosis spread beyond the local lesions and the leaf became totally necrotic (Fig. 3A). At a later stage, necrosis was seen spreading upward along the stem (Fig. 3B), and finally an entire shoot became necrotic (Fig. 3C). Necrotic lesions sometimes appeared on the upper, non-inoculated leaves (data not shown). Dot-blot analyses indicated that TMV did not spread, remaining confined to the site of infection on the inoculated leaf. This was corroborated by the inability to transfer TMV from these areas to *N. glutinosa* leaves by mechanical inoculation. Hence, T7-derived silencing brought about a phenotypic change with respect to the spread of necrosis, but the virus remained localized. The resultant phenotypes resembled those reported by Dinesh-Kumar et al. (2000) following DNA mutations in the *N* gene. Thus, DNA mutations in *N* and RNA silencing of *N* gave rise to similar phenotypes. At this point, it is worth noting that necrosis localization was uncoupled from virus localization.

Molecular verification of *N*-RNA silencing

An NBS region appears in many *R*, as well as other genes. However, NBSs carry motifs made up of several amino acids, and there is hardly any nucleotide homology among the various NBSs. Indeed, BLAST analysis of the cloned *N*-NBS segment did not result in homology to any sequence in the database, thus corroborating *N*-gene-specific silencing.

Quantitative RT-PCR analysis was carried out with primers designed to amplify a segment of *N*-NBS. RT-PCR was conducted as already described for *Ve*. Expression of the housekeeping gene (in this case 18S-rRNA) indicated similar levels of RNA in all samples (Fig. 4). Figure 5 (panels A and B) demonstrates that with similar levels of RNA templates in assays of both non-transformed and NBS-transformed plants, the level of *N*-NBS mRNA accumulation was considerably reduced in the transformed plants.

Effect of TMV infection on *N* silencing

TMV infection stimulates *N* expression in locally infected leaves as well as in the upper, virus-free, non-necrotic leaves of *N*-carrying tobacco (Levy et al. in press). TMV infection of *N*-silenced plants reversed this situation: the level of *N mRNA* in leaves after TMV infection was further reduced relative to non-inoculated pT7*N*-transformed plants. (Fig. 5C). Thus, a higher degree of silencing occurred in leaves

which (prior to silencing) harbored elevated levels of *N mRNA*. Indeed, high levels of mRNA promote the perpetuation of RNA silencing (Sela, unpublished data; Nishikura, 2001; Vaistij et al., 2002).

DISCUSSION

This paper demonstrates the silencing of endogenous genes by the T7-derived approach. The accompanying paper (Meir et al.) confirms that the *N*-gene is silenced at the RNA level, by presenting nuclear run-on transcription assays and demonstrating the presence of *N*-siRNA in the nuclei of silenced plants, as well as methylation of the silenced gene in its coding region. The aforementioned paper also suggests that various metabolic activities leading to T7-derived silencing may be confined to the nucleus. Since *GUS* is transcribed and its transcripts are detected in the nucleus (Zeitoune et al., 1999), its silencing could be attributed to cytosolic degradation of *GUS*-RNA, i.e. classical RNA silencing. Alternatively, inadequate transcription from pT7 in a eukaryotic system, or an inherent characteristic of this particular transcript, might have prevented its transport to the cytosol, resulting in nuclear confinement and non-expression, namely silencing. The presently described T7-derived silencing of endogenous genes rules out the second hypothesis. The T7 system is able to co-suppress homologous native genes whose transcripts originated from native plant promoters and were functional prior to application of the T7 system. The, T7-derived silencing system thus engenders a unique and active mechanism of RNA silencing and is not a technical or accidental phenomenon confined to the silencing of an exogenous gene. The accompanying paper suggests that T7-derived silencing differs from the RNAi type of silencing in several criteria. The present paper rules against a merely physical confinement theory, and suggests that active mechanisms are involved in T7-derived silencing.

RT-PCR analysis revealed that even when the level of silencing was sufficient to engender a new phenotype, the pertinent mRNA level, though considerably reduced, was not totally abolished. This may explain the various degrees of silencing. In the *Ve* case, silenced plants segregated into two general groups: most died following *Verticillium* infection, but some diagnosed as *Verticillium*-diseased

survived, although they remained stunted. In the case of *N*, various degrees of necrosis spread were observed.

The theory of hypersensitive reaction (HR) maintains that necrosis (programmed cell death) is associated with pathogen localization; however, a cause-and-effect relationship has not yet been determined (Dangl et al., 1996; Heath,1998). In the case of TMV localization in tobacco^{NN}, a viral component is required for necrosis. Expression of the helicase domain of the 126-kDa protein of TMV in transgenic plants engenders necrosis in tobacco (Abbink et al., 1998; Erickson et al., 1999). However, necrosis *per se* is not the cause of virus localization (Wright et al., 2000). *N* silencing brought about the spread of necrosis, but TMV remained localized. Hence, the necrosis and virus localization phenomena were uncoupled. Uncoupling of virus localization from necrosis was also demonstrated by Levy et al. (in press).

The degree of RNA silencing is often enhanced when the level of mRNA is higher (Sela, unpublished data; Nishikura, 2001; Vaistij et al., 2002). Previously (Levy et al., in press), we showed that TMV infection considerably stimulates *N* expression in tobacco^{NN} (about 30-fold in locally infected leaves and 130-fold in upper, TMV-free leaves of a plant locally infected with TMV). The results shown in Figure 5 support this finding: the order of the degree of silencing was proportional to the level of the expressed *N mRNA*. Leaves with the highest levels of *N mRNA* (upper leaves of a plant locally infected with TMV) exhibited the strongest silencing (much less *N mRNA* accumulation). The same situation, albeit to a lesser degree, was observed in locally infected leaves (data not shown).

METHODS

Plants and plant transformations. Tomato (*Lycopersicon esculentum*) plants of the cultivar VF-36 were used throughout. This cultivar carries the *Ve*1 gene for resistance to *Verticillium dahliae* and its transformation is relatively easy. Tomatoes were first transformed with the aforementioned binary plasmid harboring the gene for T7-pol, and checked for T7-pol expression by northern and western analyses (data not shown). These transformed plants became kanamycin-resistant. Another set of tomato plants were transformed with a plasmid carrying part of the *Ve*1 gene (bases 545-976; GenBank accession no. AF272366) between pT7 and the T7 and *nos* terminators, as already described. This fragment was designated pT7*Ve*. T7-pol-transformed plants

were crossed with plants transformed with pT7*Ve*. Progeny plants, resistant to both kanamycin and hygromycin, were selected. The presence of the pertinent *Ve* and *T7-pol* sequences in the resultant doubly transformed plants was confirmed by PCR.

The tobacco (*Nicotiana tabacum*) cultivar Samsun NN (tobacco^{NN}) was used throughout. This cultivar carries the *N* gene (introduced from *N. glutinosa*), conferring TMV localization by restricting infection to sites around the point of virus entry. Following TMV inoculation, necrotic local lesions also develop. However, the necrotic lesions do not superimpose the viral lesion, and the latter is larger than the necrotic one (Wright et al., 2000).

The tobacco cultivar was first transformed with a binary plasmid carrying the gene for T7 RNA polymerase (T7-pol) placed between the 35S constitutive promoter and the *nos* terminator. A nuclear localization signal was placed in front of the *T7-pol* gene, and expression of T7-pol was confirmed as described for tomato. The binary plasmid conferred kanamycin resistance on transformed plants. Another binary plasmid carrying a part of the nucleotide-binding site (NBS) domain of the *N* gene (bases 911-1250, GenBank accession no. U15605) placed between the T7 promoter (pT7) and the T7 terminator, followed by the *nos* terminator, was constructed and designated pT7*N*. This plasmid conferred hygromycin resistance on transformed plants. Plants carrying 35S-T7-pol were further transformed with the binary plasmid carrying pT7*N*. Plants resistant to both kanamycin and hygromycin were selected, and the presence of the T7-pol gene and the NBS sequence in these doubly transformed plants was checked by PCR.

Extraction of nucleic acids. For RNA extraction, tissue samples were collected, immediately frozen in liquid nitrogen, and then kept at –80°C. Total RNA was extracted from them using the RNeasy Plant Minikit (Qiagen), including QIAshredder spin columns. RNase-free DNase was then added to the column and after a 40-min incubation with the DNase, the RNA was eluted with RNase-free water. About 3 μg RNA was obtained from 100 mg of leaf tissue. DNA was extracted according to Bernatzky and Tanksley (1986).

Quantitative RT-PCR. The following primers were used for quantitative RT-PCR analyses. Primers for *N*-gene (NBS domain) detection were:
5' GATTATGGGGATCTGGG 3' and 5' CCATTACCAAACCAATC 3' (forward

and reverse primers, respectively; bases 911-927 and 1250-1233; accession no. U15605). Primers for *Ve*1 detection were: 5' TCAACCTCTTTCACATTTCCC 3' and 5' TGCCATTGTTTGCTACCG 3' (forward and reverse primers, respectively; bases 1576-1596 and 1981-1964; accession no. AF272366). Primers for tobacco 18S rRNA were: 5' AGGAATTGACGGAAGGGCA 3' and 5' GTGCGGCCCAGAACATCTAAG 3' (forward and reverse primers, respectively; bases 1142-1161 and 1466-1446; accession no. AJ236016). Primers for tomato actin were: 5' CACCATTGGGTCTGAGCGAT 3' and 5' GGGCGACAACCTTGATCTTC 3' (forward and reverse primers, respectively; bases 910-930 and 1160-1141; accession no. U60482).

For quantitative PCR, measured amounts (25 ng for *N*-gene, *Ve*1 and tomato-actin gene expression analysis and 50 pg for tobacco 18S rRNA) of total RNA extract were subjected to RT-PCR with the pertinent primers. Samples were drawn at three cycle intervals and analyzed by gel electrophoresis. The first cycle showing a visible band was indicative of the relative amount of RNA and was correlated to the internal control of the respective housekeeping gene.

Absence of residual DNA in the RNA template preparation was confirmed by the lack of product in RT-PCR assays performed without reverse transcriptase prior to the quantitative analysis.

Biological tests for *Ve*1 silencing. Maintenance or loss of *Verticillium* resistance in tomato cv. VF-36 was checked. Cultures of *V. dahliae* race 1 were maintained on Czapek's solution agar (3 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4.7H_2O$, 30 g sucrose, 15 g agar in 1 L) for 8 days at 28°C. Conidia were separated from mycelia by filtration through cheesecloth. Tomato seeds were treated with 3% sodium hypochlorite and seeded in vermiculite; 10 days later, the seedlings were removed and washed. The roots were immersed in a conidial suspension (2×10^6 conidia/ml) for 1 min and the seedlings were re-planted in sterilized sand and kept in a greenhouse at 22-24°C for up to 35 days. Susceptible plants usually became stunted within a week, and later collapsed. Xylem browning (visible following cross sectioning of shoots) was indicative of *Verticillium* infection. To ascertain that the cause of the symptoms was indeed *Verticillium*, shoots were sliced and placed on a semi-selective medium (200 g potato extract, 20 g glucose, 0.25 g chloramphenicol per 1 L). Developing mycelia were microscopically defined as *Verticillium*. The

transformation of *Verticilliium*-resistant VF-36 tomatoes into *Verticillium*-susceptible plants was indicative of *Ve*1 silencing.

Observing phenotypic changes following *N* silencing. Following TMV inoculation, tobacco plants were visually screened for the spread of necrosis. The presence or absence of TMV in plant areas to which necrosis had spread was tested by dot-blot assays and by re-inoculation of the tissue extracts onto *N. glutinosa* leaves. Necrosis spread was indicative of *N* silencing and was confirmed by quantitative RT-PCR.

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LEGENDS FOR FIGURES

Figure 1. Phenotypic demonstration of T7-derived silencing of *Ve1* in tomatos. Right row: *Verticillium*-inoculated VF-36 tomato plants. Left row: *Verticillium*-inoculated pT7V*e*-transformed VF-36 tomatoes. The picture was taken 10 days post-inoculation.

Figure 2. Quantitative RT-PCR confirmation of *Ve1* silencing. Samples were drawn from the PCR assay at three-cycle intervals. Top panel shows results of actin amplification. A: RNA was extracted from pT7*Ve*-transformed VF-36 tomato leaves. B: RNA was extracted from non-transformed VF-36 tomato leaves. In both cases an amplified band appeared at cycle 30 (lane 8) indicating equal amounts of RNA in both preparations. Bottom panel shows the results of *Ve1* amplification. Samples were drawn as described for actin. A: Amplification of RNA from pT7*Ve*-transformed VF-36 tomato leaves. B: Amplification of RNA from non-transformed VF-36 tomato leaves. An amplified band appeared in the assay of the non-transformed plants at cycle 24 (lane 6) and of the pT7Ve-transformed plants at cycle 36 (lane 11), indicating a reduced amount of *Ve1* RNA in transformed plants.

Figure 3. Phenotypic demonstration of T7-driven silencing of the tobacco *N* gene. Panel A: Necrosis spread in locally TMV-infected leaf. The right leaf is from a non-transformed tobacco^{NN} plant, exhibiting typical TMV-engendered local lesions. The left leaf is from pT7*N*-transformed tobacco^{NN}, exhibiting spread of necrosis throughout the leaf and the petiole. Panel B: Spread of necrosis along the shoot of pT7*N*-transformed tobacco^{NN}. Panel C: Spread of necrosis to upper leaves of pT7*N*-transformed tobacco^{NN}, engendering necrotic lesions there.

Figure 4. Quantitative RT-PCR for 18S-rRNA employing as templates the same RNA extracts described in Figure 5 (internal control for Figure 5). The first visible band appeared in cycle 15 (lane 2) of all samples, indicating similar amount of RNA in all samples.

Figure 5. Quantitative RT-PCR confirmation of *N* silencing in tobacco. Amplification of the *N*-NBS domain. Samples were drawn as described in the legend to Fig. 2. The plants in panels A and B were not inoculated with TMV. Panel A: RNA was extracted from non-transformed tobacco^{NN} leaves. A band was first visible at cycle 21 (lane 2). Panel B: RNA was extracted from pT7*N*-transformed tobacco^{NN}. A band was first visible at cycle 30 (lane 5), indicating a reduced amount of *N* RNA in transformed plants. Panel C: Same as in panel B, however, RNA was extracted from an upper, uninoculated leaf 72 h after TMV inoculation of a lower leaf. A band was first visible at cycle 39 (lane 8).

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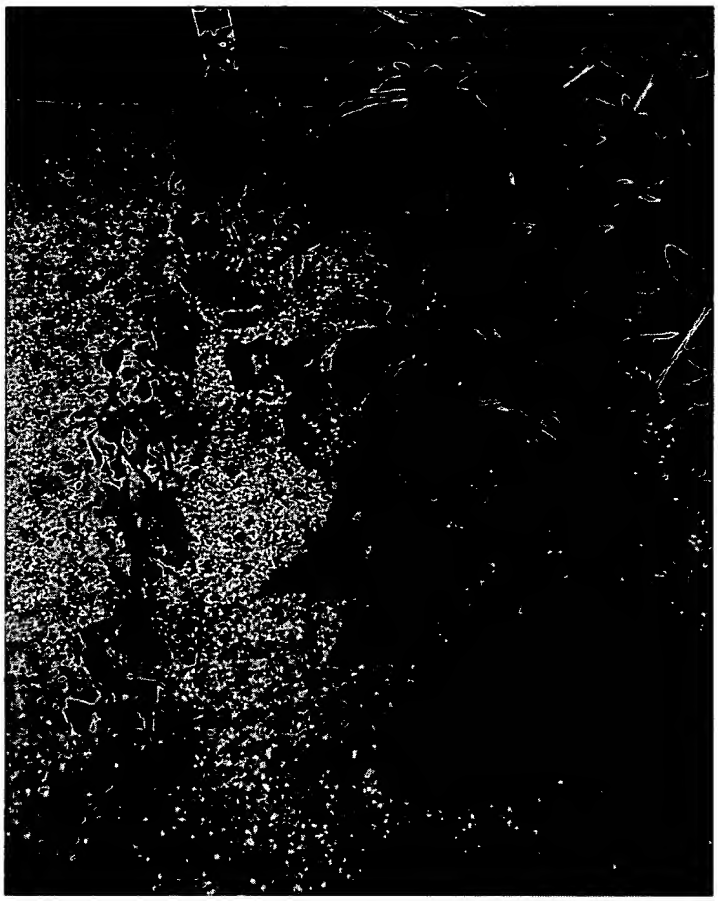

Figure 1

Appendix B, Levy et al. [1]

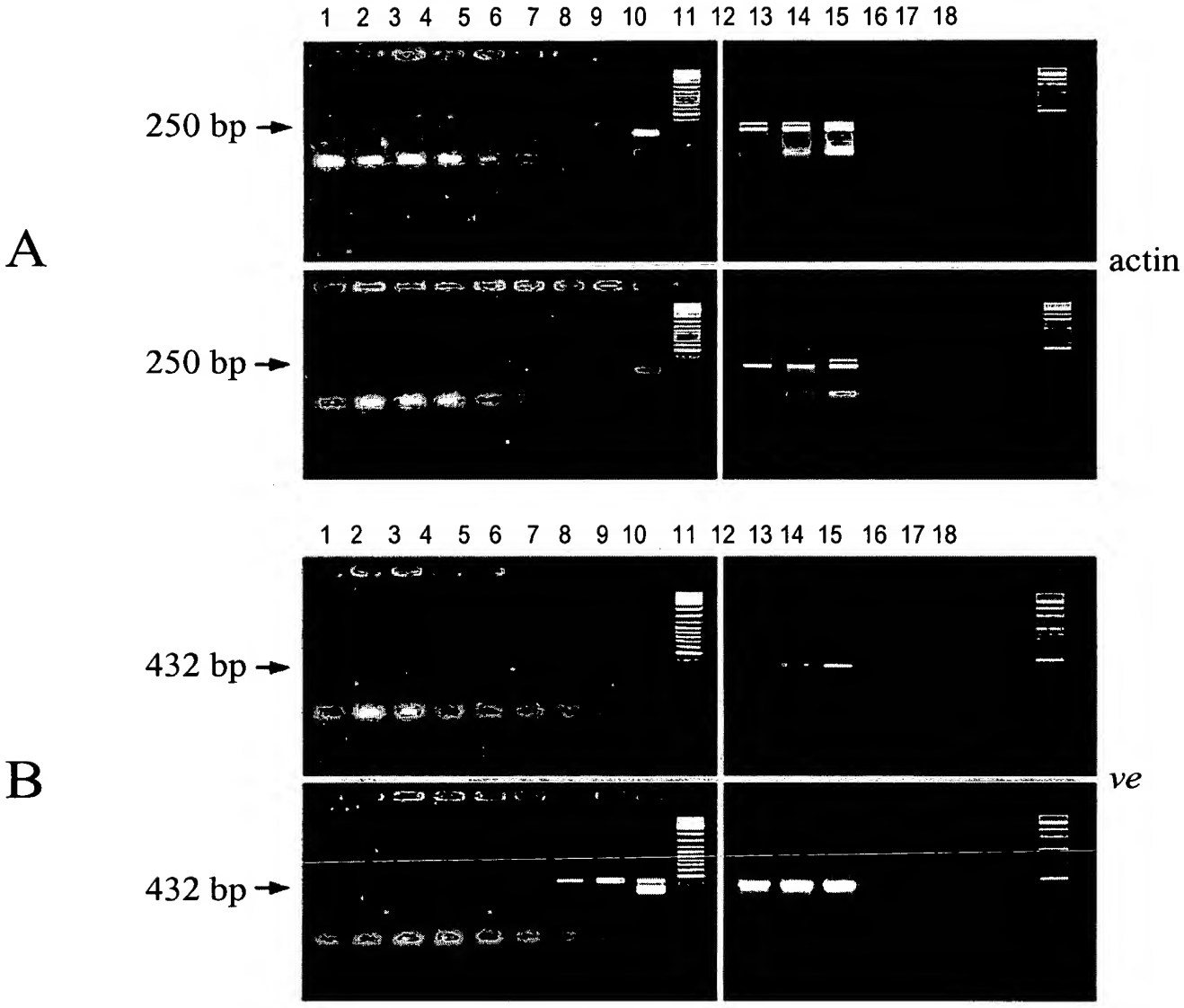
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
250 bp
A
actin
250 bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
432 bp
B
ve
432 bp

Figure 2

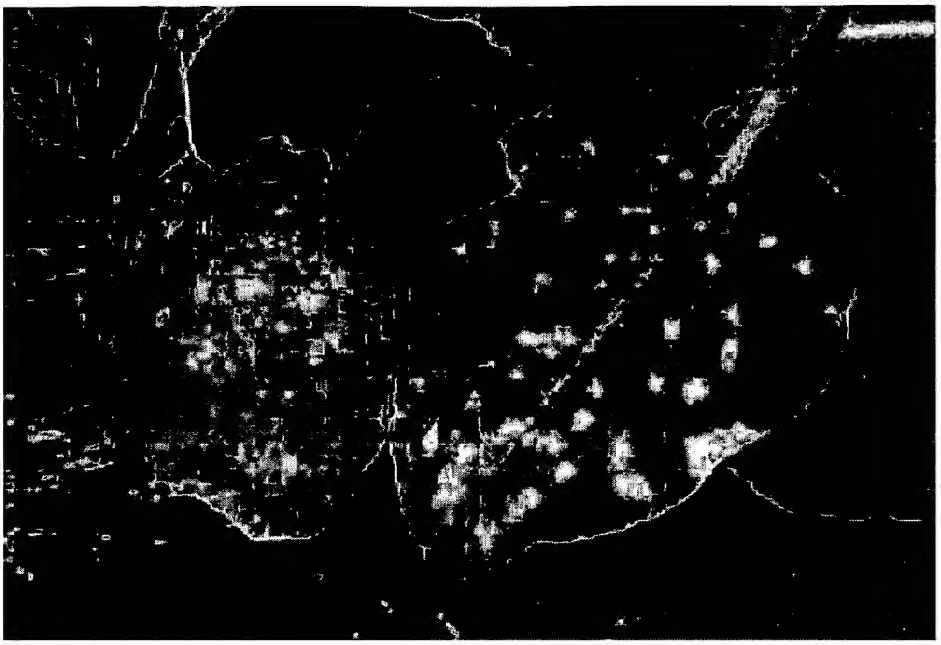

Figure 3A

Figure 3B

Appendix B, Levy et al. [3]

Figure 3C

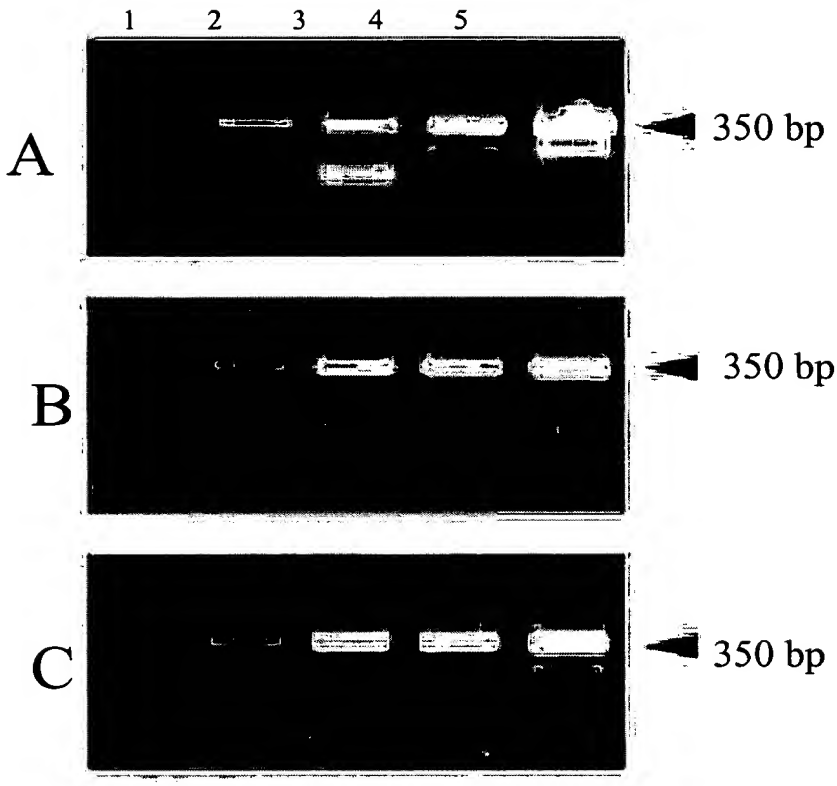

Figure 4

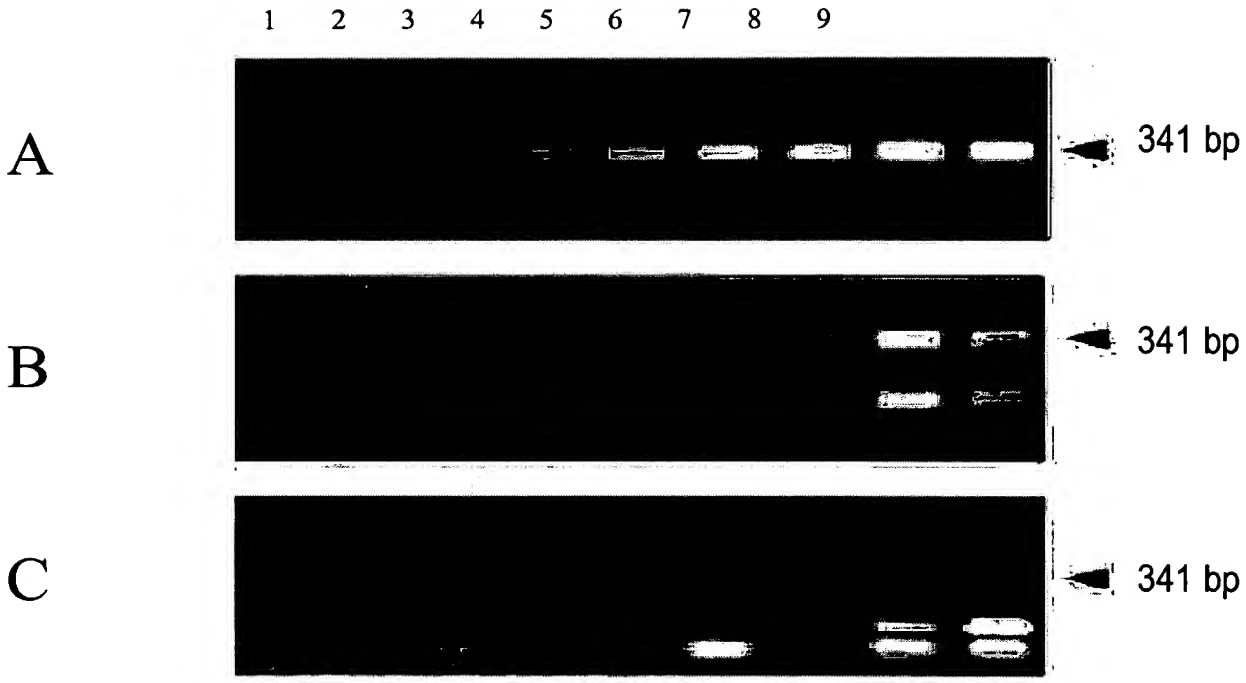

Figure 5

Appendix C

A Unique RNA-Silencing System in Plants

Tal Meir, Michal Levy, Yuval Peretz, Ophir Meir, Orit Edelbaum, and Ilan Sela[1].

The Hebrew University of Jerusalem, Robert H. Smith Institute for Plant Science and Genetics, Faculty of Agriculture, Rehovot 76100, Israel.

[1]Corresponding author. E-mail sela@agri.huji.ac.il; telephone +972-8-9489377; fax +972-8-9473402.

The corresponding author is responsible for distribution of materials integral to the findings presented in this article.

Applicants: Ilan Sela et al.
Serial No.: 09/889,821
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Exhibit 4

A Unique RNA-Silencing System in Plants

Tal Meir, Michal Levy, Yuval Peretz, Ophir Meir, Orit Edelbaum, and Ilan Sela[1].

The Hebrew University of Jerusalem, Robert H. Smith Institute for Plant Science and Genetics, Faculty of Agriculture, Rehovot 76100, Israel.

ABSTRACT

We previously demonstrated that a sequence of nucleotides placed downstream of the promoter for T7 RNA polymerase and introduced into transgenic plants harboring and expressing the gene for T7 RNA polymerase is transcribed (as indicated by nuclear run-on transcription assays), but not expressed as mRNA or protein (Zeitoune et al., 1999). We also demonstrated that the T7-derived system silences two endogenous genes: the tobacco *N* gene, conferring TMV resistance, and the tomato *Ve*1 gene, conferring resistance to the fungus *Verticillium dahliea* (Levy et al., accompanying paper). Here we demonstrate that the T7-derived silencing system is very efficient, practically silencing 100% of the tested plants. Several features characteristic of the consensus paradigm of RNA silencing are not detected in the T7-derived silencing system. We could not detect any siRNA in total RNA extracts, the silencing signal was not transduced across grafts, and the silencing suppressor HC-pro did not overcome T7-derived silencing. However, the silenced genes were methylated at their coding regions, pertinent siRNAs were detected in nuclear extracts and dicer activity in silenced plants was enhanced. We tentatively suggest that either the T7-derived silencing system is confined to the nucleus or that cytoplasmic siRNA is rapidly transported into the nucleus, impairing cytoplasmic siRNA-derived functions.

INTRODUCTION

RNA silencing down-regulates gene expression by degrading RNAs of designated sequences, arresting translation of a designated mRNA, or engendering transcriptional gene silencing. Although several aspects of the mechanisms underlying RNA silencing are still vague, a consensus pattern is emerging (recently reviewed by Cogoni and Macino, 2000; Voinnet, 2002; Agrawal et al., 2003; Pickford and Cogoni, 2003; Yu and Kumar, 2003; Matzke et al.,

2004). The first step in RNA silencing is the appearance of double-stranded RNA (dsRNA; also referred to as RNAi) carrying the sequence, or parts thereof, of the gene to be silenced (Fire et al., 1998; Thimmons and Fire, 1998; Bass, 2000; Sijen et al., 2001). In response to dsRNA accumulation, the plant (as well as other organisms) reacts by activating its RNA-silencing pathway(s). A set of RNase III-type enzymes (dicers) digest the dsRNA to small (21- to 26-nucleotide long) dsRNA fragments designated siRNAs (for example: Hamilton and Baulcombe, 1999; Zamore et al., 2000; Caplen et al., 2001; Keting et al., 2001; Llave et al., 2002). In addition to direct involvement in the process of specific RNA degradation, various roles are (tentatively) assigned to siRNAs, among them long-distance, sequence-specific signaling for silencing in other tissues, and perpetuation of cleavage of the designated target RNA (Sijen et al., 2001). SiRNAs bind to a protein complex designated RISC (Martinez et al., 2002) and their strands are separated by a RISC-associated helicase. The resultant single-stranded RNA (ssRNA) fragments guide RISC to matching sequences of the target mRNA (Lipardi et al., 2001), bringing about the cleavage of the latter. In plants, an additional route also occurs. An RNA-dependent RNA polymerase (RdRp) uses the siRNAs as extension primers for RNA elongation, dsRNA is re-formed (Wassenegger and Pelissier , 1998; Dalmay et al., 2000; Horiuchi et al., 2001; Nishikura, 2001), and silencing is perpetuated. All of these processes are believed to take place in the cytoplasm (Zeng and Cullen, 2002; Cerutti, 2003).

In plants, long-distance signal transduction of silencing, enabling the transport of a silencing signal across a graft, has been reported (Palauqui et al., 1997). RNA viruses provide their own replicase, and produce dsRNA as an intermediate in the replication process. Yet, normally, viral expression is not totally silenced in the affected host. Plant viruses carry genes for silencing suppression, for example, the potyviral HC-pro (Kasschau and Carrington, 1998; Anandalakshmi et al., 1998) and the cucumber mosaic virus (CMV) 2b protein (Lucy et al., 2000),. Some viral suppressors interact with siRNA or interfere with its production or stability (Mallory et. al., 2002; Silhavy et al., 2002; Ye et al., 2003).

RNA silencing involves nuclear activities as well. In plants, several post-transcriptionally silenced genes are methylated at their coding region (as opposed to promoter methylation in transcriptional silencing). This methylation is RNA-dependent, and siRNA has been suggested to play a guiding role in the process (Jones et al., 1999; Plissier and Wassenegger, 2000; Morel et al., 2000; Wassenegger, 2000, Bender, 2004). In addition, microRNA precursors have been found in the nucleus (Lee et al., 2002; Lund et al., 2004). Gene-specific changes in chromatin have also been reported. Methylation of histone 3 by histone H3 methyltransferase (Tamaru and Selker, 2001; Jackson et al., 2002), presumably

mediated by short RNAs, leads to the formation of heterochromatin at the promoter of the target gene, causing transcription inhibition (Zilberman et al., 2003). Cross-talk between post-transcriptional and transcriptional silencing has also been demonstrated for plants (Fojtova, et al., 2003). The aforementioned silencing suppressor, CMV 2b protein, has been localized to the nucleus (Lucy et al., 2000), indicating its intervention in a nuclear silencing process.

It has been known for some time that normal developmental processes in different organisms are mediated by siRNA-like short RNAs, i.e. short temporal RNAs, microRNAs and similar short RNAs. In these cases, short, non-coding RNAs are transcribed and partially processed. These precursors are then transported to the cytosol where they are processed to 21- to 22-bp fragments by the cytosolic dicer (Lund et al., 2004). However, dicer-like proteins were found in the nucleus of *Arabidopsis thaliana* (Papp et. al., 2003) and the dicer-like Drosha, initiating microRNA processing, is localized in the nucleus (Lee et al., 2003). These short RNAs regulate gene expression by either initiating cleavage of the target RNA or interfering with translation from the pertinent mRNA (Lee et al., 1993; Olsen and Ambros, 1999). Developmental regulation by siRNA-like microRNAs has been recently reported at an accelerated pace (for example, Yang et al., 2000; Grishok et al., 2001; Paddison et al., 2002; Storz, 2002; Bartel, 2004). Nuclear siRNA-like structures are probably products of a different type of dicing of imperfect RNA duplexes (Matzke et al., 2004).

For biotechnological purposes, RNA silencing can be engendered experimentally. Artificial introduction of dsRNA may silence a gene carrying a homologous sequence (Fire et al., 1998; McManus et al., 2002). More often, cells are transformed with a sense:antisense construct, and placing a short intron between the two opposing sequences considerably increases silencing efficiency (Waterhouse et al., 1998). Silencing can also be artificially engendered via viral infection (virus-induced gene silencing; VIGS). Placing part of an endogenous gene in a virus-vector provides, as part of the viral replicative form, a dsRNA section of the endogenous gene, leading to silencing (Ruiz et al., 1998).

Our laboratory has developed a unique and very efficient method for RNA silencing (Zeitoune et al., 1999; Levy et al., accompanying paper). Plant cells are transformed to express the RNA polymerase of the bacteriophage T7 (T7-pol). A sequence placed in these plants under the control of the T7 promoter (pT7) is then silenced at the RNA level. T7-derived silencing was obtained in all double-transformed plants. We were also able to silence, by the T7 approach, endogenous genes (the tobacco *N* gene and tomato *Ve*1 gene; Levy et al., accompanying paper) at success levels of over 80%. We were able to induce TMV recovery in tobacco with a short, 70-base sequence (data not shown).

The present paper corroborates previous findings that T7-derived silencing is a type of RNA silencing. However, in this system, several parameters associated with the aforementioned paradigm of the silencing pathway in plants were not detected. We conclude that T7-derived silencing is a unique form of RNA silencing in plants: RNA-silencing-associated components are detected in the nucleus, and at the same time, depletion of cytosolic siRNAs averts cytosolic siRNA-dependent activities.

RESULTS

T7-derived silencing is an efficient and stable post-transcriptional event

We transformed tobacco plants with the gene for T7-pol (under the constitutive 35S promoter) and confirmed *T7-pol* expression by northern and western analyses (data not shown). Other plants were transformed with the gene for GUS placed downstream of pT7. The presence of *GUS* was confirmed by PCR and Southern blot analyses. Northern and enzymatic activity tests indicated (as expected) that *GUS* is not expressed from pT7. We crossed 50 *T7-pol*-transformed plants with 50 *pT7-GUS*-transformed plants (in each case we selected plants originated from 50 independent transformation events). This resulted in 2500 types of double transformants harboring the two aforementioned constructs. A hundred plants were randomly selected and confirmed by PCR to carry both *T7-pol* as well as *GUS*. None of the 2500 doubly transformed plants expressed *GUS*, as evidenced by the lack of GUS activity upon staining. Non-expression of *GUS* could not be attributed to inadvertent *GUS* modification introduced during the process of cloning, since, as described later, the same *GUS* construct was expressed under the 35S promoter and the enzyme was active. Segregation of silencing was checked through at least 4 generations of self-pollinated silenced plants. T7-derived silencing was found to be stable, and every progeny along the generations, which carried both constructs, was GUS-silenced.

The aforedescribed type of silencing is not limited to *GUS* or tobacco. Our accompanying paper (Levy et al.) demonstrates T7-derived silencing of two endogenous genes: the tobacco *N* gene and the tomato *Ve*1 gene.

Further on in this paper we show that the mechanism underlying T7-derived silencing deviates from that of the mainstream RNA-silencing pathway. Therefore, it was crucial to check not only that *GUS* is transcribed in the described *GUS*-silencing system (Zeitoune et al.,

1999) but that silencing of an endogenous gene is also post-transcriptional. To that end, we performed nuclear run-on transcription tests with *N*-silenced plants. Figure 1 clearly shows that *N* transcription does occur in silenced tobacco^{NN} plants, hence non-expression of *N* (like *GUS*) is due to a post-transcriptional event.

No siRNA is detected in total RNA extracts from T7-derived silenced plants

We were not able to detect any *GUS*, or *N*-related siRNA in the total RNA extracts of plants which were fully silenced for *GUS* or *N* expression by the T7-derived method. As a positive control, we transformed tobacco with the gene for green fluorescent protein (GFP), under the 35S promoter, and then silenced GFP expression via a typical RNAi technique: we introduced an intron-spliced hairpin of a GFP sequence to the GFP-expressing plant, as described in Methods. In this case, *GFP*-related siRNA was detected (Fig. 2), and *GFP* expression was silenced (Fig. 3B). Contrary to the classical silencing of GFP, T7-derived silencing is not accompanied by the appearance of the pertinent cytosolic siRNA; alternatively, the amount of siRNA produced may be unusually low, rendering it undetectable (Fig. 2). SiRNA could not be detected in these samples even by the more sensitive RNase protection assay (data not shown).

T7-derived silencing is not suppressed by a viral silencing suppressor

The suppression of RNA silencing by viral-directed silencing suppressors is a well-documented phenomenon. The HC-pro cistron of potyviruses has been shown to be a powerful suppressor of silencing (for example Anandalakshmi et al., 1998; Kasschau and Carrington, 1998). Figure 3 shows that plants silenced for *GFP* expression by the typical spliced-intron hairpin method regained *GFP* expression following inoculation with potato virus Y (PVY). In contrast, plants *GUS*-silenced by the T7-derived method did not regain expression following PVY inoculation (Fig. 4). Further transformation of the *GUS*-silenced plants with the PVY cistron for HC-pro did not alleviate silencing either (data not shown).

Lack of long-distance signaling in T7-derived silencing

In plants, long-distance silencing signals engender silencing in non-silenced scions upon grafting on silenced rootstocks. This was not the case in the T7-derived silencing system (Table 1). Doubly transformed plants, silenced for *GUS*, were grafted with scions harboring and expressing 35S-*GUS* without silencing *GUS* expression in the scions. Reciprocally, GUS expression also occurred when the 35S-*GUS* plants served as rootstocks on which the *GUS*-silenced doubly transformed plants were grafted. To rule out the possibility that lack of signaling between rootstock and scion was due to inept grafting technique, we grafted TMV-infected scions on healthy plants, and demonstrated the virus' spread across the graft. We also demonstrated that in the case of intron-spliced hairpin silencing of *GFP*, a long-distance signal is transduced across the graft, engendering silencing in GFP-expressing scions (data not shown).

SiRNA is present in the nuclei of silenced plants

Nuclei were isolated from *GUS*- and *N*-silenced plants as described in Methods. Nuclear RNA was extracted and subjected to the siRNA-detection assay. In both cases, a specific (21-23 bp) band was detected with the pertinent probe (Fig. 5) either by the conventional Northern assay for siRNA (Hamilton and Baulcombe, 1999) or by the more sensitive RNase protection assay described in Methods. T7-derived silencing is therefore associated with the appearance of short RNAs in the nuclei of the silenced plants, while their accumulation in the cytosol is nil or negligible. The nuclear siRNA is double-stranded as indicated by its reaction with both sense and antisense probes in the RNase protection assays (Fig. 6). A control of an untransformed plant probed with a segment of the gene for tobacco actin did not react with the sense probe and only the expected-size transcript reacted with the antisense probe. SiRNA was not detected in this control plant. Plants transformed with 35S-*GUS* also produce ds-siRNA as expected from highly-expressing transgenic plants.

DNA methylation

The restriction enzymes *Hpa*II and *Msp*I cleave at the same restriction site (CCGG). However, the first is methylation-sensitive, while the latter cleaves methylated sites as well. Genomic DNA from non-transformed tobacco^{NN} plants and from *N*-silenced plants was cleaved with one of the two enzymes, electrophoresed, and subjected to Southern analysis. A labeled fragment of the *N* gene, carrying the NBS domain (Levy et al., accompanying paper) served as a probe (Fig. 7). The *Msp*I and *Hpa*II cleavage patterns of DNA from non-transformed plants were identical. However, the pattern of the *Hpa*II-cleaved DNA from silenced plants differed from that cleaved by *Msp*I. An extra band, representing a longer fragment, was observed in the *Hpa*II cleavage pattern, indicating that, at least in part, a CCGG sequence was methylated at the NBS domain of *N*. To confirm silencing-derived methylation and determine methylation density, we mapped cytosine methylation by the bisulfite method as described in Methods. This test was performed on DNA from *GUS*-silenced plants. A dense DNA methylation was observed at the *GUS* coding region, and only a few unmethylated cytosines were detected (Fig. 8). As a control for the validity of the bisulfite mapping of cytosine methylation we have repeated the experiment with a *GUS*-carrying plasmid. In this case C to T and G to A shifts were observed all along (data not shown). This analysis also indicated that in silenced plants cytosine methylation is not restricted to GC or GNC sites, and any cytosine residue can be methylated

Stimulation of dicer activity in silenced plants

We have performed a dicer assay as described in Methods with protein extracts from silenced (by the T7-derived method) silenced and non-silenced plants, following up the production of a

small dsRNA (ca. 21 bp) from a RNA duplex. Cleavage of dsRNA by RNAse III served as a control and marker of the size of siRNA. Dicer is an inherent activity in plants and siRNA was produced by extracts from silenced as well as non-silenced plants (Fig. 9). However, higher levels of siRNA were produced with extracts from T7-silenced plants. It is worth noting that nuclear protein extracts from *GUS*-silenced plants did not cleave dsRNA (lane 7). Since siRNA was found in the nuclei, this may indicate that nuclear siRNAs are transported from the cytoplasm or that nuclear dicers do not recognize perfect RNA duplexes.

DISCUSSION

Plants which are doubly transformed with 35S-*T7-pol* and pT7-*GUS* undergo *GUS* transcription, but no *GUS* mRNA or GUS activity can be detected therein. By definition, this phenomenon is a type of RNA silencing. It could be argued that this is only a matter of incorrect transcription from pT7, leading to the appearance of aberrant, or non-processed *GUS* transcripts, and does not constitute a general mechanism beyond the reported individual case. However, specific silencing-associated nuclear activities were also shown to be associated with T7-derived silencing. In addition, endogenous genes (capable of proper transcription and processing) can be silenced by the T7 method (Levy et al., accompanying paper). Therefore, following T7-derived silencing, a cascade of activities must take place, engendering the silencing of normally expressed genes as well. Therefore this type of silencing (although artificial) is of a general nature, and not merely the result of anomalous transcription in an individual case.

This report shows that the silencing mechanism in the case of T7-derived silencing differs from that of the consensus paradigm for RNAi-derived silencing. It is very efficient

(80-100% of the engineered plants become silent), it does not involve detectable levels of cytosolic siRNA, it does not signal for silencing across a graft, and it is not suppressed by a viral silencing-suppressor. At present, only three activities known to be associated with the mainstream model for RNA silencing have been shown to occur in the T7 system: DNA methylation of coding regions of the silenced gene, accumulation of nuclear, silencing-specific, short RNAs, and stimulation of dicer activity.

Signal transduction of silencing is believed to be associated with siRNA (Mallory et al., 2003), and viral suppressors are believed to intervene in siRNA accumulation (Llave et al., 2000). Lack of signal transduction and the non-responsiveness to HC-pro suppression may be due to depletion of cytosolic siRNA.

The current model for nuclear siRNA-like production is a nuclear cleavage by a dicer-like enzyme of target transcripts of an imperfect duplexes (Palatnik et al., 2003) producing precursors (70-110 b long) for farther dicer cleavage in the cytosol where the final 21-26-long siRNA appears (Lee et al., 2003). RNAi-derived siRNAs are then transported to the nucleus

On the other hand, dicer activity was enhanced in cytosolic fractions (but not in nuclear extracts) of T7-derived silenced plants. Taken together, it is conceivable that the T7-derived system stimulates a very efficient mechanism of transporting siRNA from the cytoplasm to the nucleus. The nucleus becomes, in fact, a sink for siRNA, and siRNA-directed nuclear activities are enhanced. At the same time, siRNA-directed cytosolic activities are impaired, diminishing its guiding role for signal transduction. Alternatively, the entire T7-derived silencing process may be confined to the nucleus (and inaccessible to HC-pro intervention). The absence of dicer activity in nuclear extracts and its presence in the cytosolic fraction favors the sink theory. The silencing suppressor HC-pro interferes with siRNA production or destabilizes siRNA (Mallory et al., 2002). In the present case, siRNA

may become inaccessible to HC-pro due to its rapid transport to the nucleus, therefore this silencing suppressor does not affect silencing.

METHODS

Plants and transformation. Two tobacco (*Nicotiana tabacum* L.) varieties were used. The *GUS*-silencing system was constructed in the variety SR1, and the *N*-silencing system was constructed in the variety Samsun^{NN}, carrying the *N. glutinose N* gene for tobacco mosaic virus (TMV) localization.

The GUS-silencing system is described in Zeitoune et al. (1999). In brief, tobacco SR1 plants were Agro-transformed with a binary vector carrying the RNA polymerase (RNA-pol) gene from the bacteriophage T7, constitutively expressed from the 35S promoter and harboring a gene for a selectable marker (*NPTII*). Another set of plants were similarly transformed with a construct carrying the *GUS* gene downstream of the T7 promoter (pT7) and the same selectable marker. *T7-pol*-carrying plants were crossed with pT7-*GUS*-carrying plants. The resultant doubly transformed plants were silenced for *GUS* (this article and Zeitoune et al., 1999).

The *N*-silencing system is described in Levy et al. (accompanying paper). Tobacco Samsun^{NN} plants, carrying and expressing *T7-pol*, were re-transformed with a binary vector harboring a 341-bp DNA segment, corresponding to the NBS region of *N* (under pT7 control) along with a selectable marker conferring resistance to hygromycin. Plants resistant to both kanamycin and hygromycin were selected and PCR-tested for *T7-pol* and pT7-*N*-NBS, as described in the aforementioned paper.

Tobacco SR1 was transformed with the gene for GFP, placed downstream of the 35S promoter.. For RNAi-type silencing, a spliced hairpin construct (described below) was introduced into *GFP*-expressing plants under the control of the 35S promoter.

GUS assays. GUS expression was assayed by staining leaves with chloro-3-indolyl glucuronide (X-gluc). Quantitative GUS expression was measured with methylumbelliferyl glucuronide (MUG). Both assays were carried out according to Jefferson (1987). The MUG assay was normalized for protein content. Protein was determined according to Bradford (1976).

The improved nuclear run-on assay. A rapid method for the isolation of nuclei was adopted from Kanazawa et al. (2000). However, to avoid non-nuclear contamination, the nuclei were separated on a Percol gradient, and the run-on assays were performed as described by Zeitoune et al. (1999).

The construct for *GFP* silencing. The construct was a generous gift from S. Michaeli (Bar Ilan University, Ramat-Gan, Israel). It consisted of a 200-bp segment from the sequence of the *GFP* gene (accession # U87974, bases 251-449), followed by a 106-bp segment of an intron of the *CCA1* gene (accession # U79156, bases 3724-3380) and the same 200-bp *GFP* segment in the opposite orientation. The construct was introduced into the binary vector pBIN under the control of the 35S promoter. Tobacco SR1 plants, carrying and expressing *GFP*, were Agro-transformed with this construct for *GFP* silencing.

SiRNA assay. Leaf tissue or purified nuclei were homogenized in Tri Reagent (Sigma) and their RNA was extracted according to the manufacturer's protocol. The presence of siRNA was determined using two methods: (a) Northern assays for the detection of siRNA as described by Hamilton and Baulcombe (1999) with the following modifications: the extracted RNA was not size-fractionated, and electrophoresis was conducted in 10% polyacrylamide, 8 M urea gels. Prehybridization and hybridization were performed in 60% formamide at 42°C. Following hybridization, the membranes were washed with 2X SSC at 60°C. (b) RNase protection assay (Zeitoune et al, 1999) was used to detect low levels of siRNAs. Radioactive fragments of the pertinent gene (200-500 bases each) were transcribed *in vivo*, hybridized with the target RNA, subjected to RNase A and RNase T1 digestion and electrophoresed on the aforementioned acrylamide-urea gels. Sense and antisense probes were *in-vitro*-transcribed from a *GUS*-carrying plasmid, and a plasmid carrying a 241-bp segment of the NBS region of the *N* gene.

Dicer assay. Leaf proteins were extracted by homogenizing on ice 0.5 g leaf tissue in 1 ml protein lysis buffer (30 mM HEPES pH 7.4, 100mM potassium acetate, 2 mM magnesium acetate, 5 mM DTT, 100 μM phenylmethanesulfone fluoride, 1mM benzamidine), followed by 15 min centrifugation at 12,000 g. Protein content was determined according to Bradford (1976). Dicer activity was carried out as described by Liu et al. (2003) The obtained supernatant fluid (5 μg protein per assay, determined as described above) served as a dicer source. Cloned *GUS* was transcribed in both orientations and transcripts of both polarities

were allowed to hybridize as described by Liu et al., (2003). The resultant (ca. 1400 bp) dsRNA served as substrate for RNase III (Biolab) producing 21 bp-long dsRNAs.

DNA-methylation assays. Genomic DNA was extracted according to Bernatzky and Tanksley (1986). Mapping of 5'-methylated cytosine residues in the DNA by the bisulfite method was performed according to Clark et al. (1994). Genomic DNA was cleaved with *EcoR*I and *Hind*III (neither enzyme cleaves within the *GUS* transgene) and the cleaved DNA was submitted to the aforementioned bisulfite treatment. Following bisulfite conversion, most of the *GUS* transgene sequence was amplified by PCR followed by nested PCR. Conversion of cytosine to thymine is expected wherever the cytosine is not methylated. Therefore, degenerated PCR primers were designed. The PCR primers were: 5' ATT GAT YAG YGT TGG TGG GA 3' and 5' TRC RRT CRC RAR TRA ARA TC 3'. The nested PCR primers were: 5' AAAAYGGYAAGAAAAAGYAG 3' and 5' CAAAATCRRCRAAATTCCATAC 3'. The product of the nested PCR was 1205-bp long (between positions 1920 and 3125 of the sequence in GenBank, accession # M14641).

In addition, methylation assays were conducted by restriction analysis, basically according to Aufsatz et al. (2002). DNAs from non-silenced and *N*-silenced tobacco^{NN} were cleaved with *Hpa*II (a methylation-sensitive enzyme) and *Msp*I (methylation insensitive). Both enzymes cleave at the same site (CCGG). Following cleavage, the two patterns were compared to determine possible methylation of a CCGG site.

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LEGENDS TO FIGURES

Figure 1. Nuclear run-on assay with nuclei isolated from *N*-silenced tobacco^{NN}. The assay was carried out as described in Methods. The source of cloned DNA placed in each dot is marked. Bluescript DNA served as a negative control for unrelated DNA.

Figure 2. Detection of siRNA in total RNA extractions. Samples from GFP-silenced, GUS-silenced and *N*-silenced plants were electrophoresed on the same gel and transferred onto a membrane. The membrane was then cut and the segments hybridized with the pertinent probes (*GFP*, *GUS*, and *N*). Panel A. Positive control: Detection of siRNA in *GFP*-silenced plants (silencing was engendered by the intron-spliced hairpin method). The size of the band is 21 bp. Panel B (lanes 1, 2): Patterns obtained with samples from GUS-silenced plants. (lane 3): end-labeled size markers. Panel C: Patterns obtained with samples from *N*-silenced plants.

Figure 3. Silencing and suppression of silencing of *GFP*. Confocal microscope images are presented. Panel A: GFP fluorescence in tobacco (SR1). Panel B: Decreasing fluorescence (silencing) following the introduction of an intron-spliced hairpin into the plant shown in A. Panel C: Reappearance of fluorescence (suppression of silencing) of the plant shown in panel B following infection with potato virus Y (PVY). Panel D: Non-transformed tobacco. Panel E: PVY-infected, non-transformed tobacco.

Figure 4. *GUS* silencing is not suppressed by HC-pro. Tobacco (SR1) leaf sections were stained for GUS. The single leaf section on top is a positive control from a GUS-expressing plant. Leaf sections from six *GUS*-silenced plants, following staining for GUS, are shown in the top two rows. Leaf sections in the bottom two rows were taken from the same plants 4 weeks following inoculation with PVY.

Figure 5. Detection of siRNA in nuclei of silenced plants. Lanes 1-8: results of RNase protection assays. Lanes 7, 8: results of Northern analysis for siRNA. Lane 1: Nuclear RNA from untrasformed plants reacted with an antisense-oriented probe for actin. Lane 2: same as in lane 1, reacted with a sense-oriented probe for actin. Lane 3: Nuclear RNA from *N*-silenced plants reacted with an antisense-oriented probe. Lane 4: same RNA as in lane 3 reacted with a

sense-oriented probe. Lane 5: Nuclear RNA from a *GUS*-expressing transgenic plant reacted with a sense-oriented probe. Lane 6: same RNA as in lane 5 reacted with an antisense-oriented probe. Lane 7: Nuclear RNA from *GUS*-silenced plants reacted with a sense-oriented probe. Lane 8: same RNA as in lane 7 reacted with a sense-oriented probe. Lane 9: Northern detection of siRNA in nuclei of *GUS*-silenced plants. Lane 10:: same as lane 9, but nuclear RNA was extracted from untransformed plants.
This is a composite figure of several gels, hence the siRNA positions are not precisely aligned. RNase III-cleaved dsRNA served as size marker in every gel (as in Fig. 8), enabling the identification of the 21 bp position.

Figure 6. Evidence of DNA methylation. DNA was extracted from non-silenced and from N-silenced tobacco^{NN}. The DNA was first cleaved with *EcoR* I (which does not cut within the gene) and then with *Hpa* II or *Msp* I. The resultant DNA fragments were electrophoesed, blotted and reacted with a radioactive probe of the NBS segment of *N*.
Lane 1: pattern of *Msp* I-cleaved DNA from untransformed plant. Lane 2: pattern of *Hpa* II-cleaved DNA from the same plant as in lane 1. Lane 3: pattern of *Msp* I-cleaved DNA from a *N*-silenced plant. Lane 4: pattern of *Hpa* II-cleaved DNA from a *N*-silenced plant. The arrow points at a longer extra band appearing in *Hpa* II-cleaved DNA from *N*-silenced plants.

Figure 7. Mapping of cytosine methylated sites in a silenced plant. DNA from *GUS*-silenced plants was treated with bisulfite and further analyzed as described in Methods. The sequence obtained after the bisulfite treatment is presented. Most C residues were not altered indicating that they were methylated. The position of some unmehtylated C residues are indicated by shifts from C to T or G to A (bold letters). A control mapping of unsilenced *GUS* (in a plasmid) indicated that none of the C residues in the presented sequence was methylaed (data not shown).

Figure 8. Stimulation of dicer activity in silenced plants. Dicer activity was tested as described in Methods. The appearance of siRNA is indicative of dicer activity. Lane 1: positive control; dsRNA was cleaved with RNase III. In the other lanes dsRNA was cleaved with protein extracts from the indicated plants. Lane 2: from GFP-silenced plants (silencing was by the spliced hairpin method). Lane 3: from *GUS*-silenced plant (silencing was T7-derived). Lane 4: From a plant transformed with the gene for T7-RNA polymerase (a parent of the *GUS*-silenced plant). Lane 5: From a plant transformed with the gene for GUS under

the T7 promoter (another parent of the *GUS*-silenced plant). Lane 6: From untransformed tobacco SR1. Lane 7: From nuclei of *GUS*-silenced plants.

Table 1: Fluorometric measurements of GUS activity in *GUS*-expressing scion/rootstock following grafting of a *GUS*-silenced tissue. GUS analysis was carried out by the MUG assay described in Methods.

Scion/rootstock	GUS activity (nmole/h per mg protein) at various times after grafting			GUS staining in the 35S-*GUS* part*
	1 month	2 months	3 months	
35S-GUS/SR1**	337	320	347	+
SR1/SR1	0	0	0	-
35S-GUS/35S-GUS	335	365	230	+
DT/DT***	0	0	0	-
35S-GUS/DT	395	405	496	+
35S-GUS/DT	225	79	120	+
35S-GUS/DT	110	140	125	+
35S-GUS/DT	60	301	395	+
35S-GUS/DT	60	350	515	+
35S-GUS/DT	350	325	340	+
35S-GUS/DT	221	254	267	+
DT/35S-GUS	220	190	240	+
DT/35S-GUS	98	73	102	+
DT/35S-GUS	130	160	144	+
DT/35S-GUS	152	235	251	+
DT/35S-GUS	206	227	230	+

*All plants signed by + were stained positively with X-gluc at all times.
**Untransformed tobacco
***DT delineates doubly transformed plants silenced for *GUS* expression.

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actin
Kanamycin
Rubisco
No DNA
N-DNA
(NBS domain)
No DNA
Bluescript

Figure 1

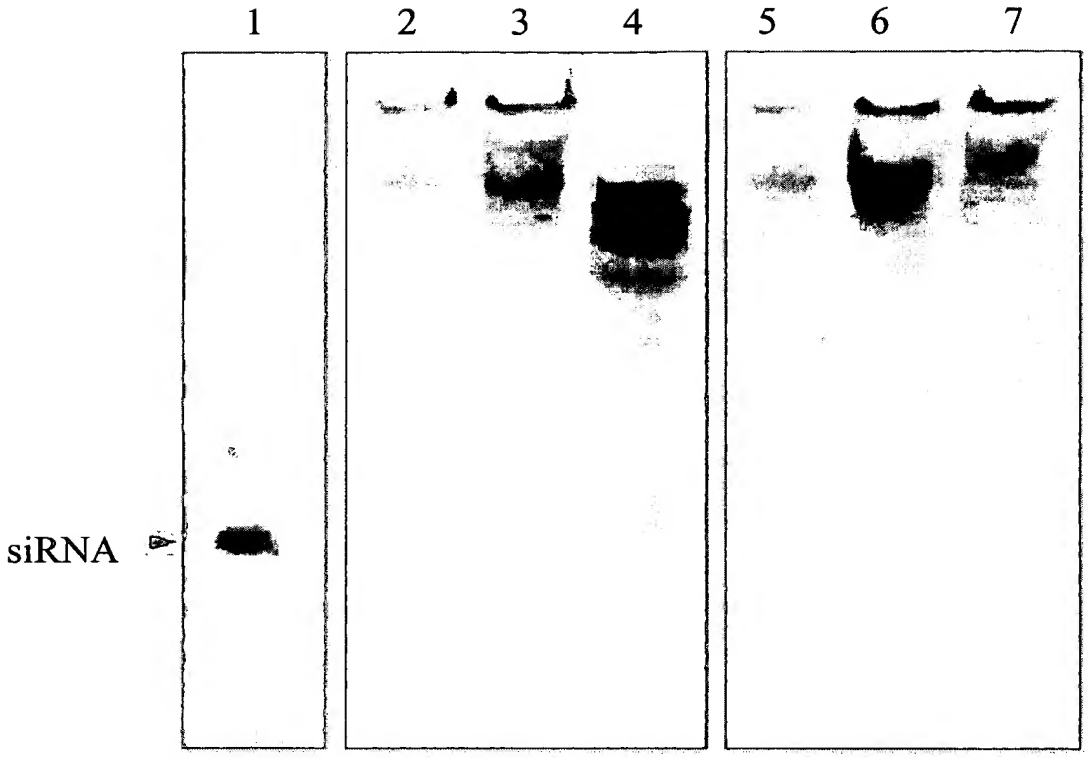


Figure 2

Figure 3A Figure 3B Figure 3C

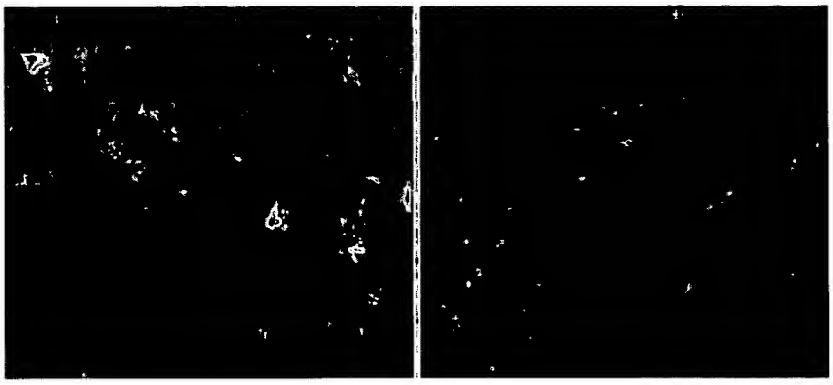
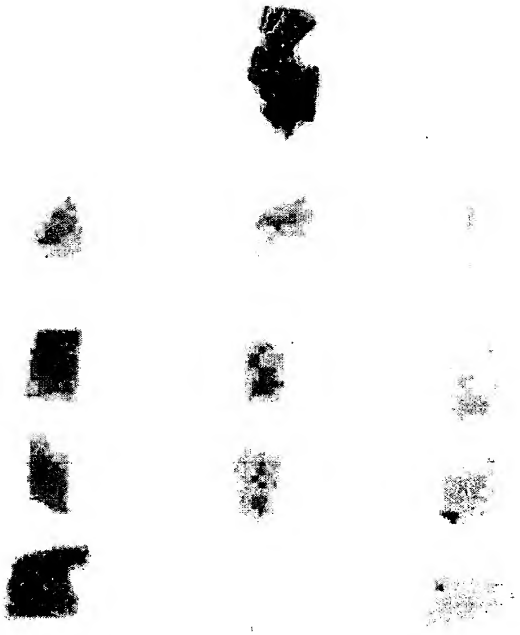


Figure 3D Figure 3E



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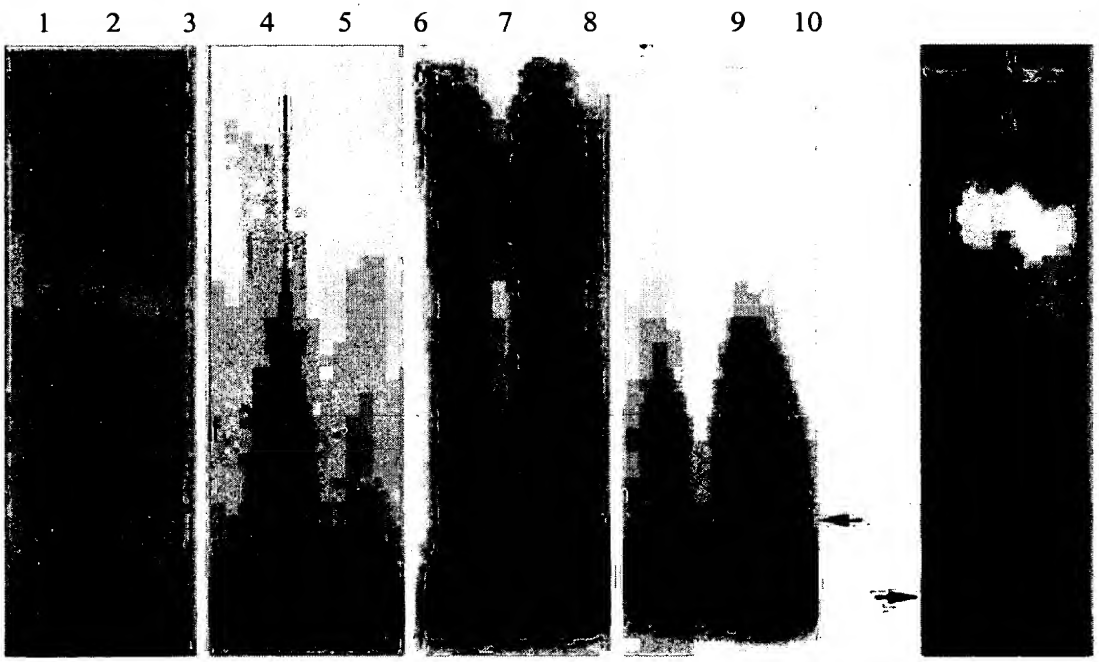

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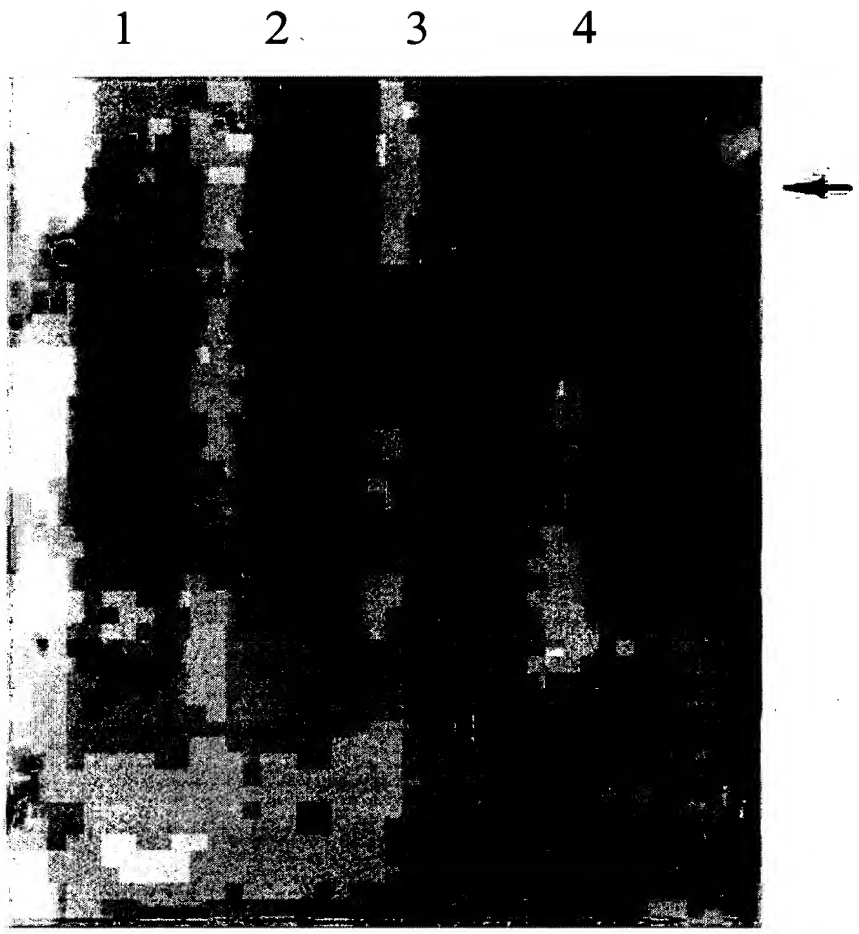

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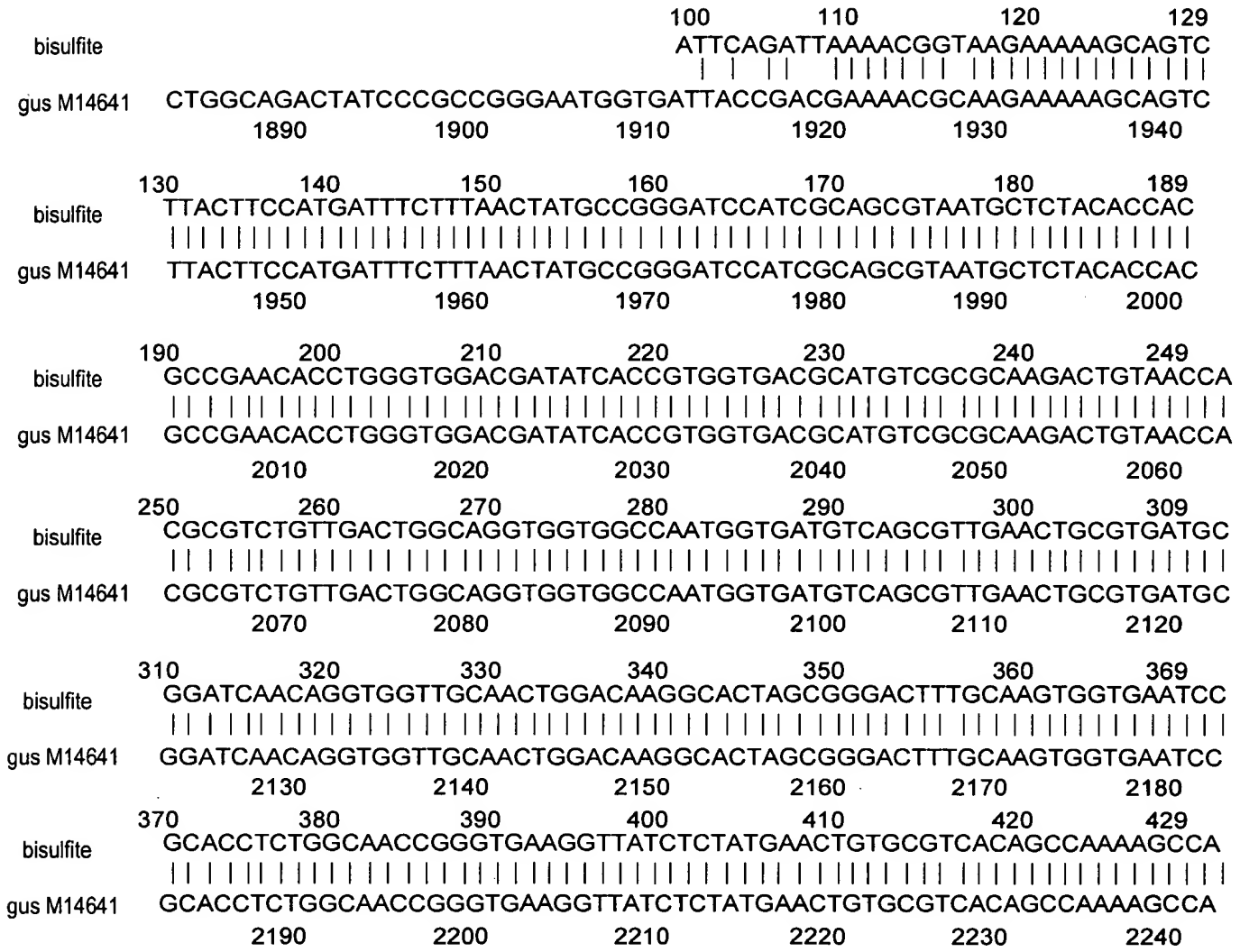
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Figure 7

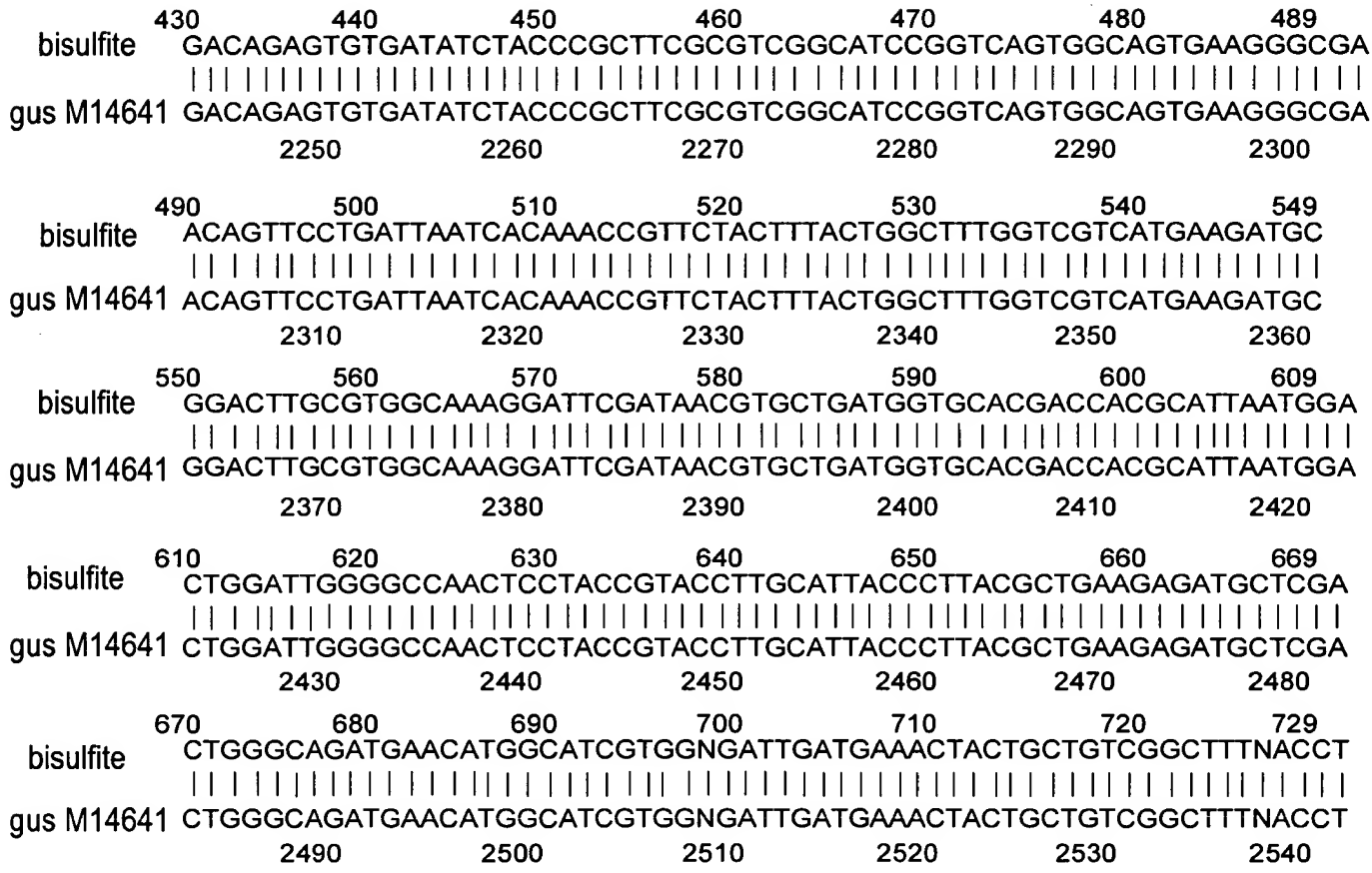

Figure 7 (cont.)

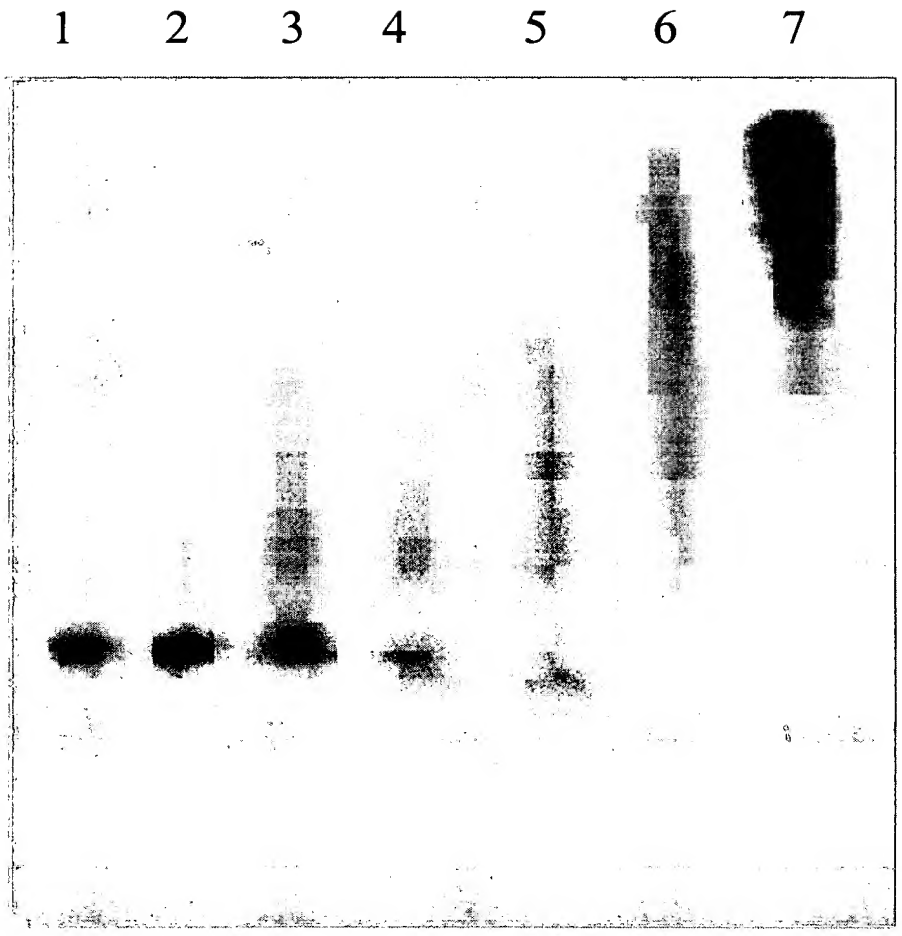

Figure 8